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Strategic Environmental Research and Development Program

Explosives Conjugation Products in Remediation Matrices: Interim Report 2

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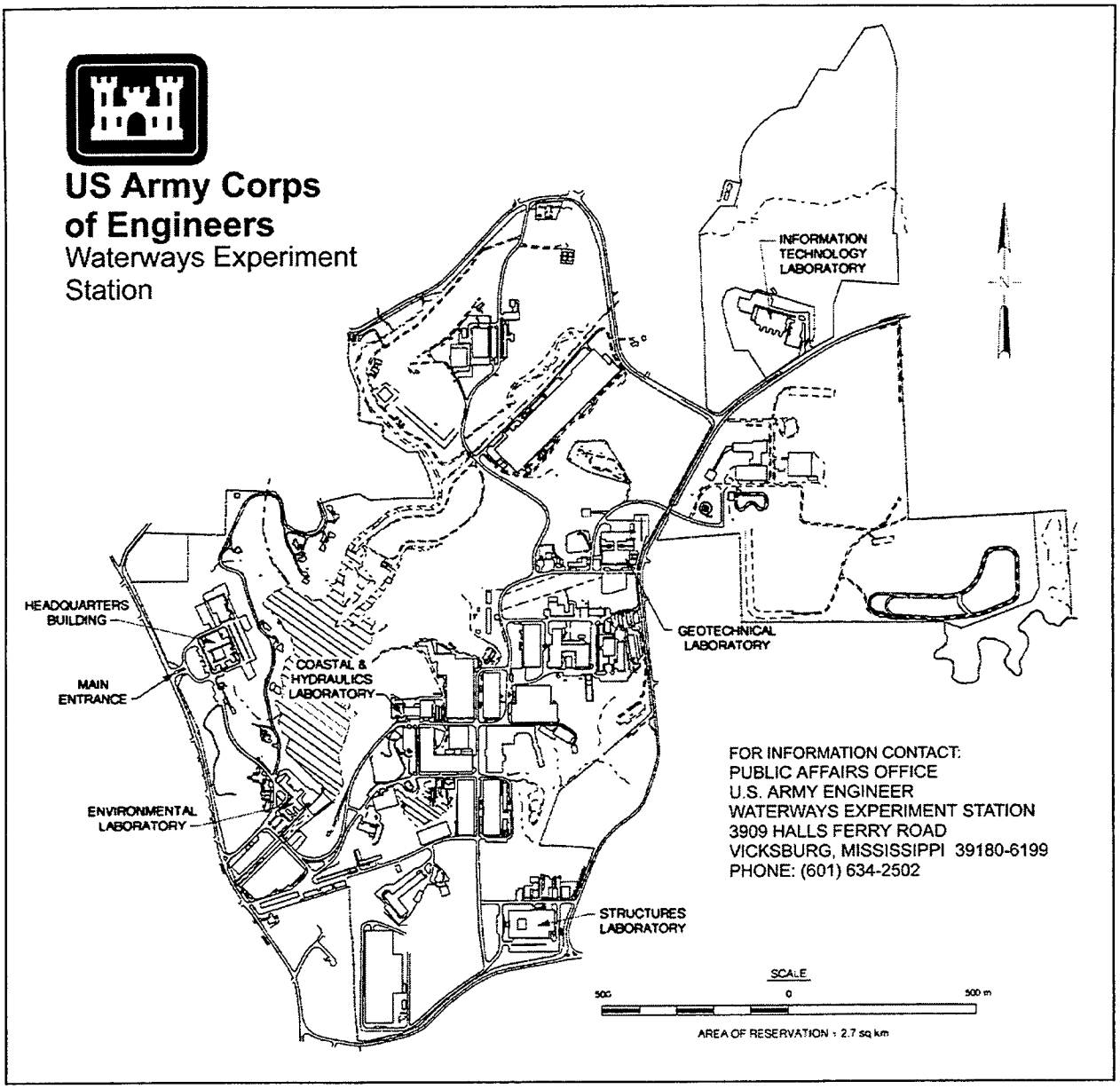

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Contents

Preface

This report was prepared by the Environmental Laboratory (EL) of the U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS, in association with the U.S. Army Cold Regions Research and Engineering Laboratory (CRREL), Hanover, NH; the U.S. Geological Survey (USGS), Golden, CO; DynTel Corporation, Atlanta, GA; and AScI Corporation, McLean, VA. The research was sponsored by the Strategic Environmental Research and Development Program (SERDP), Arlington, VA. Dr. M. John Cullinane, EL, was the WES Program manager for the SERDP. The Principal Investigator was Dr. Judith C. Pennington, EL.

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At the time of publication of this report, Director of WES was Dr. Robert W. Whalin. Commander was COL Robin R. Cababa, EN.

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1 Introduction

Background

Research from several sources indicates that the explosive 2,4,6-trinitrotoluene (TNT) becomes immobilized in soil and compost systems. Some of the earliest evidence for immobilization of TNT in soils came when radiolabeled TNT and 4-amino-2,6-dinitrotoluene (4ADNT), a transformation product of TNT, were added to soils for a plant uptake study (Pennington 1988). Comparisons of percent recoveries of ^{14}C by solvent (acetone) extraction and from complete combustion of soil revealed that an average of four times as much of the added radioactivity was recovered by combustion. Solvent extraction failed to remove the immobilized residues of TNT or of 4ADNT. These results indicated that standard analytical techniques, which require solvent extraction of the TNT and its transformation products, fail to reveal a significant quantity of immobilized contaminant.

Evidence for immobilization of TNT in compost resulted from a study in which radiolabeled TNT was added to soil prior to composting (Pennington et al. 1995). After 20 days, the compost was fractionated into solvent-extractable (ether followed by acetonitrile), cellulose, fulvic acid, humic acid, and humin components. Less than 20 percent of the added radioactivity was solvent extractable. Since TNT is much less soluble in water than in these organic solvents, this result suggested that mobilization of TNT or of its products from compost by water in the environment is limited. More than 30 percent of the added radioactivity was associated with the cellulose fraction, and more than 20 percent was associated with the humin. Cellulose is a biodegradable component of organic matter that may release the contaminant in some form, but humin is extremely recalcitrant to further degradation in the environment.

Results of these studies indicated that immobilization of TNT and/or its transformation products is a significant fate process. An understanding of the nature and mechanisms of immobilization may be very important to the develop ment of effective treatment technologies. Furthermore, if the extent of immobiliza tion in the environment approaches the extent observed in the laboratory, immobilization may exceed adsorption and degradation processes in importance as a contaminant fate process. The information generated by this research may be used to develop natural attenuation of explosives in soils and groundwater, to assess the ultimate fate of

explosives in compost, and to estimate the bioavailability of explosives in biotreatment systems.

An interim report describing results of research prior to Fiscal Year 1998 (FY98) (Pennington et al. 1997) included the results of the following research areas:

Toxicology: Cytotoxicity and mutagenicity of 2,4,6-trinitrotoluene (TNT) and its metabolites

Dialysis Partitioning: Trinitrotoluene and metabolites partitioning to humic acid

Hydrolytic Release: Hydrolytic release of bound residues from composted TNT-contaminated soil

Microbial Degradation: Microbial degradation of conjugated fractions

Surface Plasmon Resonance: Analysis of coupling affinities among TNT degradation products and humic acid based on surface plasmon resonance

Covalent Binding: Use of ^{15}N NMR to evaluate covalent bonding of TNT transformation products to surrogates for functional groups on soil humic acid molecules

Four of the research areas, toxicology, hydrolytic release, microbial degradation, and covalent binding, continued in FY98 and are the subject of this report. The following is a brief summary of results presented in the FY97 interim report.

Toxicology

This study was undertaken to examine the *in vitro* cytotoxicity and mutagenicity of TNT and several of its reduction products. Cytotoxicity assays were conducted using two cultured cell lines, Reuber H35 H4IIE rat hepatoma cells and Chinese Hamster Ovary-K1(CHO) cells. Mutagenicity of the compounds was assessed using Ames and Mutatox assays. Results indicated that TNT, 4-hydroxylamino-2,6-dinitrotoluene, 4,4', 6,6'-tetranitro-2,2'-azoxytoluene (2,2'AZOXY) and 2,2',6,6'-tetranitro-4,4'-azoxytoluene (4,4'AZOXY) were equally cytotoxic to H4IIE and CHO cells. Two other TNT transformation products, 4ADNT and 2-amino-4,6-dinitrotoluene (2ADNT), were cytotoxic to H4IIE cells, but not to CHO cells. Another transformation product, 2,4-diamino-6-nitrotoluene (2,4DANT), was noncytotoxic to either cell type. These results indicated that the transformation products of TNT mentioned above are as cytotoxic as, or only moderately less cytotoxic than, TNT *in vitro*. Results of Ames assays indicated that TNT with and without metabolic activation was slightly mutagenic to strain TA98, but not mutagenic to strain TA100. Only 2ADNT was slightly mutagenic to strain TA98 without activation. Both 2ADNT and 2,2'AZOXY exhibited mutagenicity in strain TA100 with and without activation, while 4ADNT was slightly mutagenic without activation. In Mutatox assays, TNT was mutagenic without S9 activation, 2ADNT, 4ADNT, and 2,4DANT were mutagenic both with

and without S9 activation. One significant generalization of these results is that while the two most commonly detected transformation products of TNT, 2ADNT, and 4ADNT, are equally or only slightly less toxic than the parent compound, their mutagenicity as measured by the Mutatox assay is significantly higher than that of TNT.

Dialysis partitioning

These studies were conducted to quantify the adsorption of TNT and several of its transformation products to humic acid. Equilibrium dialysis was used to determine the effects of contaminant concentration, humic acid concentration, pH, and ionic strength on the formation kinetics and on extent of interaction. Results indicated that TNT, 2,6-diamino-4-nitrotoluene (2,6DANT), and 2ADNT bind to humic acid slowly. As the concentration of humic acid increased, the extent of binding of the two compounds decreased. As pH increased, the extent of binding increased. Nearly a twofold increase in binding of TNT was observed for a five-fold increase in ionic strength. A linear model best fit the 2,6DANT isotherm data, while a Langmuir model best fit the TNT data. The maximum binding density of TNT for humic acid was 6 to 30 μM TNT per μM humic acid.

Hydrolytic release

The objectives of this study were to differentiate between bound and unbound explosives and their transformation products in composted soil, to determine the time course of metabolite evolution and binding during composting, and to evaluate various hydrolysis methods for assaying bound products. Compost samples were subjected to two hydrolysis procedures: acid (50 percent aqueous sulfuric acid) and base (0.5 M sodium hydroxide) followed by acid (concentrated sulfuric acid). Results indicated that significant quantities of identifiable aminodinitrotoluenes and diaminonitrotoluenes were released after solvent-extracted residues were subjected to acid or base hydrolysis. However, no TNT was released. The concentration of hydrolyzable metabolites reached a maximum at 10 days of composting, then decreased through 40 days. This study produced a method for removing and identifying a significant portion of the immobilized products of TNT.

Microbial degradation

An important question concerning the remediation technologies in which immobilization products are formed is long-term stability of the product. To address this concern, the biodegradability of immobilized products was examined. This study had two objectives. The first objective was to determine the ability of several common enzymes to modify TNT and/or mediate reactions between TNT and major soil organic components. These enzymes are typically active in degradation of aromatic contaminants and are common in microorganisms. The second objective was to evaluate the ability of soil microflora to mineralized radiolabeled TNT that has been immobilized in soil and/or compost. Results on the first

objective showed that none of the three enzymes, polyphenol oxidase, laccase, or polyphenol oxidase, exhibited activity against TNT. Results for the second objective showed active enrichment cultures on fractions of compost : acetonitrile extract, cellulose, fulvic acid, humic acid, and humin. These studies continue. Enrichment cultures growing on fractions from radiolabeled compost exhibited mineralization ($^{14}CO_2$) from cellulose and humic acid fractions. Further studies include examination of physiology and biochemistry of the interactions occurring between the microorganisms and the TNT-substrate products.

Surface plasmon resonance

The Biospecific Interaction Analysis System (BIA) presents a method by which coupling affinity between molecules can be assessed. This instrumental system is extremely sensitive for investigating molecular interactions at the surface of a sensor chip using *surface plasmon resonance* (SPR) to analyze the concentration of the biomolecules attached to the chip surface. The rationale was to bind the smaller molecules of TNT (or transformation products) to the surface matrix of the chip and introduce the larger humic acid molecules over the surface. If the humic acid interacted with the TNT, the kinetics could be followed by minute changes in weight to which the instrument is extremely sensitive. Since this is state-of-the-art technology requiring adaptation to procedures of this study, great care was taken to validate performance and execution of tests. Results indicated that neither TNT nor two of its transformation products, 4ADNT and 2,6DANT, could be immobilized on the chip surface. Various modifications of pH, concentration, and flow rate were tried without success. Binding of the humic acid, rather than the explosive, was also explored without success. Apparently, binding of a substrate to the chip material is chemical class specific. Protein experiments confirmed that the system functions well with "biospecific" compounds, but not with all other classes of organic compounds.

Covalent binding

Recent research using liquid and solid-state ^{15}N-nuclear magnetic resonance (NMR) has demonstrated that aniline binds covalently to isolated humic substances and to the organic matter in whole soils and peat (Thorn, Arterburn, and Mikita 1992; Thorn et al. 1996). Aniline reacts with isolated fulvic and humic acid in the presence and absence of phenoloxidase enzymes or metal catalysts. In the absence of catalysts, aniline undergoes a complex series of nucleophilic addition reactions with quinones and other carbonyl groups to form both heterocyclic and nonheterocyclic condensation products. In the presence of the enzyme or metal catalyst, aniline undergoes free radical coupling reactions together with nucleophilic addition reactions with the humic substances. Since common reductive products of TNT, i.e., 2ADNT, 4ADNT, 2,4DANT, 2,6DANT, chemically resemble aniline in functionality groups, these reductive products may mimic the coupling reactions of aniline with humic acid.

The objectives of this study were (a) to demonstrate covalent binding of 4ADNT, 2ADNT, and 2,4DANT with model carbonyl compounds, with humic substances, and with whole compost and soil, and (b) to determine the chemical lability of the bound amino residues. To demonstrate binding to model carbonyl compounds, the ^{15}N-NMR shifts (liquid phase NMR) of condensation products of amines with, e.g., 4-methylcatechol, 1,4-benzoquinone, and glucose, were measured. The spectra of product mixtures from amine reactions were also measured. Reference fulvic and humic acid (from the International Humic Substances Society) were reacted with TNT reduction products without catalysts (liquid- and solid-phase NMR). Organic matter fractions of compost were extracted and reacted with TNT reduction products. The products were analyzed by liquid- and solid-state ^{15}N-NMR. Time series interactions ranging from weeks to months were conducted to determine interactions between reduction products and both compost and surface soils. To determine the chemical lability of bound residues, exchange of the bound residues with other amines or release of the residues by hydrolytic enzymes and microbial degradation were tried. Results provided a chemical definition of a significant immobilization process for TNT and TNT transformation products.

Objectives

The broad objectives of this study were to determine the nature and mechanism of the immobilization interactions between TNT and soil components, the long-term stability, biodegradability, and toxicity of the products of those interactions, and methods for detecting products of the interactions. Specific objectives of each study are presented within each chapter. Only the toxicology, hydrolytic release, microbial degradation, and covalent binding studies continued in FY98 and are reported here.

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2 ^{15}N NMR Studies on the Covalent Binding of the Reductive Degradative Products of TNT to Humic Substances, Model Compounds, and Peat

Introduction

Recent ^{15}N NMR studies provided direct spectroscopic evidence for the covalent binding of aniline, the parent compound of the aromatic amines, to humic substances, to model compounds representing functional groups present in humic substances, and to the organic matter of whole soil and to peat. Nitrogen -15 NMR studies were therefore undertaken to confirm that the reductive degradation products of TNT also undergo covalent binding to the naturally occurring organic matter in soil and peat. Several questions were addressed: (a) Do the amines undergo nucleophilic addition reactions with organic matter to form covalent bonds? (b) What are the nature and quantities of bonds formed? (c) Do the amines differ in reactivity towards covalent bond formation (nucleophilicity), or do the types of bonds formed differ? (d) How do phenol oxidase enzyme or metal catalysts affect the reactivity of the amines and the distribution of condensation products? (e) What is the chemical stability of the covalent bonds formed under catalyzed and noncatalyzed reaction conditions?

The aminodinitrotoluenes and diaminonitrotoluenes were synthesized with ^{15}N labels in the amine positions. The product mixtures from the reaction of the amines with 4-methylcatechol (4MC) in the absence of catalysts were analyzed by liquid-phase ^{15}N NMR to assess the relative nucleophilicity of the amines toward quinones. The product mixtures from the horseradish peroxidase catalyzed reaction of 4ADNT and 2,4DANT with conniferyl alcohol, a precursor to lignin and a plausible structural entity within soil humic acid, were also analyzed by liquid-phase ^{15}N NMR. The incorporation of the amino nitrogen into the International

Humic Substances Society (IHSS) soil humic acid upon reaction with 2ADNT, 4ADNT, 2,4DANT, and 2,6DANT, in the presence and absence of horseradish peroxidase, was observed directly using a combination of liquid- and solid-state ^{15}N NMR. Lastly, the susceptibility to base and acid hydrolysis of the covalent bonds between the four amines and Pahokee peat was determined using solid-state ^{15}N NMR.

Materials and Methods

Materials

The reference soil humic acid (isolated from the mollic horizon of the Elliot Silt Loam soil, Joliet, IL), Pahokee peat (Ocachobee, FL; 44.48-percent organic C), and Suwannee River fulvic acid were purchased from the IHSS (www.ihss.gatech.edu/). The 4-amino-^{15}N-2,6-dinitrotoluene (4ADNT) and 2-amino-^{15}N-4,6-dinitrotoluene (2ADNT) were custom synthesized by Dr. Ron Spanggord, SRI International, Menlo Park, CA (Spanggord 1998). The 2,4-diamino-^{15}N 2-6-nitrotoluene (2,4DANT) and 2,6-diamino-^{15}N 2-4-nitrotoluene (2,6DANT) were custom synthesized by ISOTEC (Miamisburg, OH). Aniline, 99 atom percent ^{15}N, was purchased from ISOTEC. Horseradish peroxidase (EC1.11.1.7; 53 purpurogallin units/mg solid) was purchased from Sigma (St. Louis, MO). Birnessite was prepared by the method of McKenzie (1971). 4-Methylcatechol and conniferyl alcohol were purchased from Aldrich. (Use of trade names in this report is for identification purposes only and does not constitute endorsement by the U.S. Geological Survey or U.S. Army.)

Reactions of 4-methylcatechol with amines

Approximately 55 mg of 4ADNT, 2ADNT, 2,4DANT, or 2,6DANT were dissolved separately in 1-L pH 6 phosphate buffer with heating. After the solutions cooled to room temperature, 340 mg 4-methylcatechol (4MC) were added, and in the case of the catalyzed reactions, 100 mg horseradish peroxidase and 8 ml hydrogen peroxide. The noncatalyzed reactions were allowed to stir open to the atmosphere for 16 to 26 days, and the peroxidase-catalyzed reactions 6 to 9 days. The reaction solutions were then passed through a C18 Mega-Bond Elut cartridge (Varian Chromatography Products, Harbor City, CA) to recover the colored reaction products. In general, the colored reaction products were recovered in consecutive water and methanol elutions, the majority of material in the latter. The aqueous elution fractions were freeze dried, and the methanol elution fractions rotary evaporated.

Peroxidase catalyzed reactions of conniferyl alcohol with 4ADNT and 2,4DANT

One hundred fifteen milligrams of conniferyl alcohol, 100 mg peroxidase, and 8 ml hydrogen peroxide, respectively, were added to a solution of 100 mg 4ADNT dissolved in 2 L of pH 6 phosphate buffer. The solution was stirred for 7 days. The conditions were the same for the reaction of 2,4DANT with conniferyl alcohol, except that 200 mg of the alcohol was used. The colored reaction products were recovered on a C18 Mega-Bond Elut cartridge as described above.

Reactions of IHSS soil humic acid with amines

For reactions with the monoamines, 180-200 mg of 2ADNT or 4DANT were dissolved in 4 L of deionized and distilled water. For reactions with the diamines, 200 mg of 2,4DANT or 2,6DANT were dissolved in 2 L deionized and distilled water. For each reaction, 500 mg of the H+-saturated soil humic acid was added to 400 ml H_2O and dissolved by adjusting the pH to 6.4 with 1 N NaOH. In the case of the peroxidase-catalyzed reactions, 120 mg of the enzyme were dissolved in the humic acid solution. The humic acid solutions were added to the solutions of dissolved amine. In the case of the peroxidase-catalyzed reactions, 16 ml of hydrogen peroxide was then added to the combined solutions of amine, humic acid, and enzyme. The solutions were stirred open to the atmosphere and at room temperature for 14 to 20 days in the case of the noncatalyzed reactions and 9 to 13 days in the case of the peroxidase-catalyzed reactions. The samples were then H+-saturated again by passing the solutions through a Dowex MSC-1 cation exchange column (Dow Chemical, Midland, MI) and freeze dried. Suwannee River fulvic acid was reacted similarly with 2,4DANT and no catalyst.

Reactions of Pahokee peat with amines

Two grams of Pahokee peat was added to solutions of 200 mg each of 2,4DANT or 2,6DANT dissolved in 2.5 L of distilled deionized water, 200 mg of 4ADNT in 3 L water, and 150 mg 2ADNT in 3 L water, respectively. The solutions were sonicated for approximately 30 min to disperse the peat, stirred for 3 months open to the atmosphere, but protected from exposure to any li ght source, and then freeze dried. The peat was also reacted with 2,4DANT in the presence of 150 mg birnessite for 1 month. The freeze-dried peat samples were then dialyzed in 1,000 Dalton MW cutoff tubes to remove the unreacted free amines, which are highly colored. The dialyzed peat samples were then freeze dried again for NMR analyses and hydrolysis experiments.

Hydrolysis of reacted peat samples

The hydrolysis was performed on 0.4-g samples of conjugated peat that had not been solvent extracted. In a 22-ml glass vial, a 3.0-ml aliquot of 0.5 M NaOH was added to the sample. The vials were capped and sonicated for 18 hr in a

cooled ultrasonic bath. After the sonication with base, a 3.0-ml aliquot of ice-cold 50-percent H_2SO_4 was added. The vials were sonicated for 6 more hr in a cooled ultrasonic bath. The vials were centrifuged, and the digest supernatants containing the formerly conjugated amino transformation products were trans ferred to clean 125-ml beakers. The acid digests were neutralized by adding a 1.0 M solution of K_2HPO_4 (pH 8.5) to the digest supernatants, stopping the neutralization when the pH = 5.0. The neutralized digestion supernatants were extracted by passing the supernatants through a precleaned PoropakRDX solid-phase extraction cartridge (Millipore, Bedford, MA), following the manufacturer-supplied instructions.

The retained transformation products were eluted from the cartridges by drawing through a 5-ml aliquot of acetonitrile. The volume of the recovered acetonitrile was brought up to 10 ml by adding reagent-grade water. Analysis was performed by the RP-HPLC using an aqueous/methanol gradient as follows: start at 85/15 (vol%/vol%), ramp to 65/35 at 8 min, ramp to 42/58 at 10 min and hold for 13 min, ramp to 0/100 at 28 min and hold for 7 min, ramp down to 85/15 at 40 min and hold for 10 min before the next injection. The flow rate was 0.8 ml/minute using a Phenomenex Ultracarb 5 ODS(20) (4.6 by 250 mm, 5 = B5 m) column (Torrence, CA).

NMR spectrometry

Liquid-phase ^{15}N NMR spectra were recorded on a Varian XL300 (Varian NMR, Palo Alto, CA) or GEMINI 2000 NMR spectrometer at a nitrogen resonant frequency of 30.4 MHz using a 10-mm broad-band probe. Two sets of ^{15}N NMR spectra were recorded on the product mixtures from the reactions of 4-methylcatechol with the amines. The first spectrum was recorded using an 18,656.7-Hz spectral window (613.7 ppm), 45° pulse angle, 0.5-s acquisition time, 5.0-s pulse delay, and inverse-gated decoupling. The second was a refocused (proton decoupled) insensitive nuclei enhancement by polarization transfer (INEPT) spectrum recorded using an 18,656.7-Hz spectral window, 0.5 -s acquisition time, and delay for proton relaxation of 2.0 s. The polarization transfer time and refocusing delay were set equal to 1/4 J, or 2.78 ms (assuming $^1J_{NH}$ = 90.0 Hz), values that have been reported optimal for signal enhancement of singly protonated nitrogens. In the case of the fulvic and humic acids reacted with the amines, approximately 300 mg of the reacted samples were dissolved in 2 ml dimethylsulfoxide (DMSO)-d6. Three sets of ^{15}N NMR spectra were recorded. An INEPT spectrum was recorded using the above acquisitions parameters. An alternating compound one eighties used to suppress transients in the coil (ACOUSTIC) spectrum was recorded, chromium III acetylacetonate (100 mg) was then added to the sample, and the ACOUSTIC spectrum was rerecorded. Acquisition parameters for the ACOUSTIC sequence included an 18,656.7-Hz spectral window, 0.5-s acquisition time, 45° pulse angle, 2.0-s pulse delay, and t delay of 0.1 ms. Only the ACOUSTIC spectra recorded with the addition of the paramagnetic relaxation reagent are shown. Neat formamide in a 5-mm NMR tube, assumed to be 112.4 ppm, was used as an external reference standard for all

spectra. ^{15}N NMR chemical shifts are reported in ppm downfield of ammonia, taken as 0.0 ppm.

Solid-state CP/MAS (cross polarization/magic angle spinning) ^{15}N NMR spectra of the soil humic acids reacted with the amines were recorded on a Chemagnetics CMX-200 NMR spectrometer (Chemagnetics, Fort Collins, CO) at a nitrogen resonant frequency of 20.3 MHz, respectively, using a 7.5 -mm ceramic probe (zirconium pencil rotors). Acquisition parameters included a 30,000-Hz spectral window, 17.051-ms acquisition time, 2.0-ms or 5.0-ms contact time, 1.0-s pulse delay, and spinning rate of 3,500 Hz. The peat samples were acquired using a 2.0- or 5.0-ms contact time, 0.5-s pulse delay, and spinning rate of 5,000 Hz. Chemical shifts were referenced to glycine, taken as 32.6 ppm.

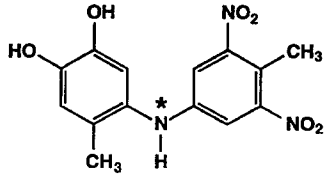
Results and Discussion

Background

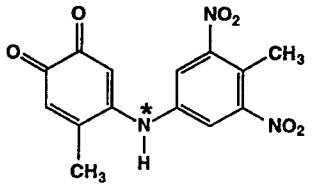
The reaction chemistry of aniline with humic substances is summarized in Figure 1. The nucleophilic addition and free radical coupling reactions are assumed to be generally applicable to the reduced nitro moieties of TNT, i.e., the amines.

Reaction of amines with 4-methylcatechol

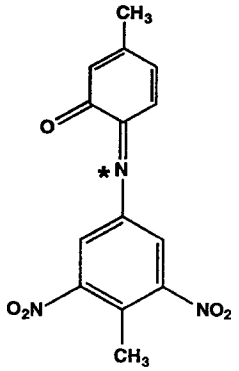
Figure 2 shows the liquid phase ^{15}N NMR chemical shifts of the amino compounds along with the pKa's of the amino groups. Figure 3 shows quantitative liquid-phase ^{15}N NMR spectra of the methanol eluates from the product mixtures of the reaction of 4-methylcatechol (4MC) with the amines in the absence of catalyst. 4ADNT reacts with 4MC to form a number of products, as indicated by the multiplicity of discrete peaks in the NMR spectrum. The peaks around 88 ppm correspond to aminohydroquinone nitrogens, whereas the peaks at around 106 ppm correspond to aminoquinone nitrogens. The minor peak at 301 ppm corresponds to imine nitrogen.



Aminohydroquinone



Aminoquinone



Imine

In the absence of catalysts, aromatic amines undergo nucleophilic addition reactions with quinone and other carbonyl groups to form heterocyclic and nonheterocyclic condensation products.

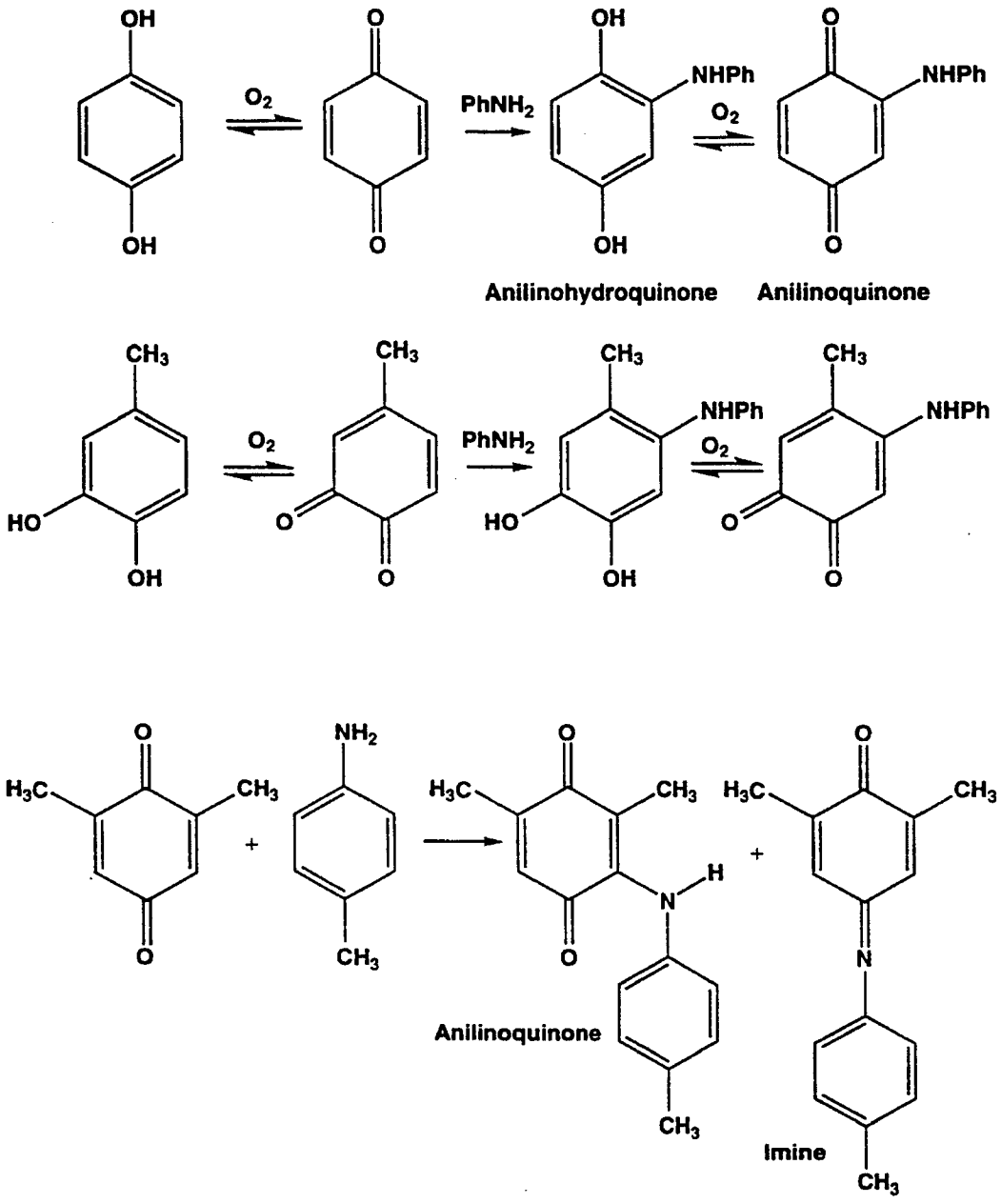


Figure 1. Reactions of aniline with organic functional groups (Sheet 1 of 5)

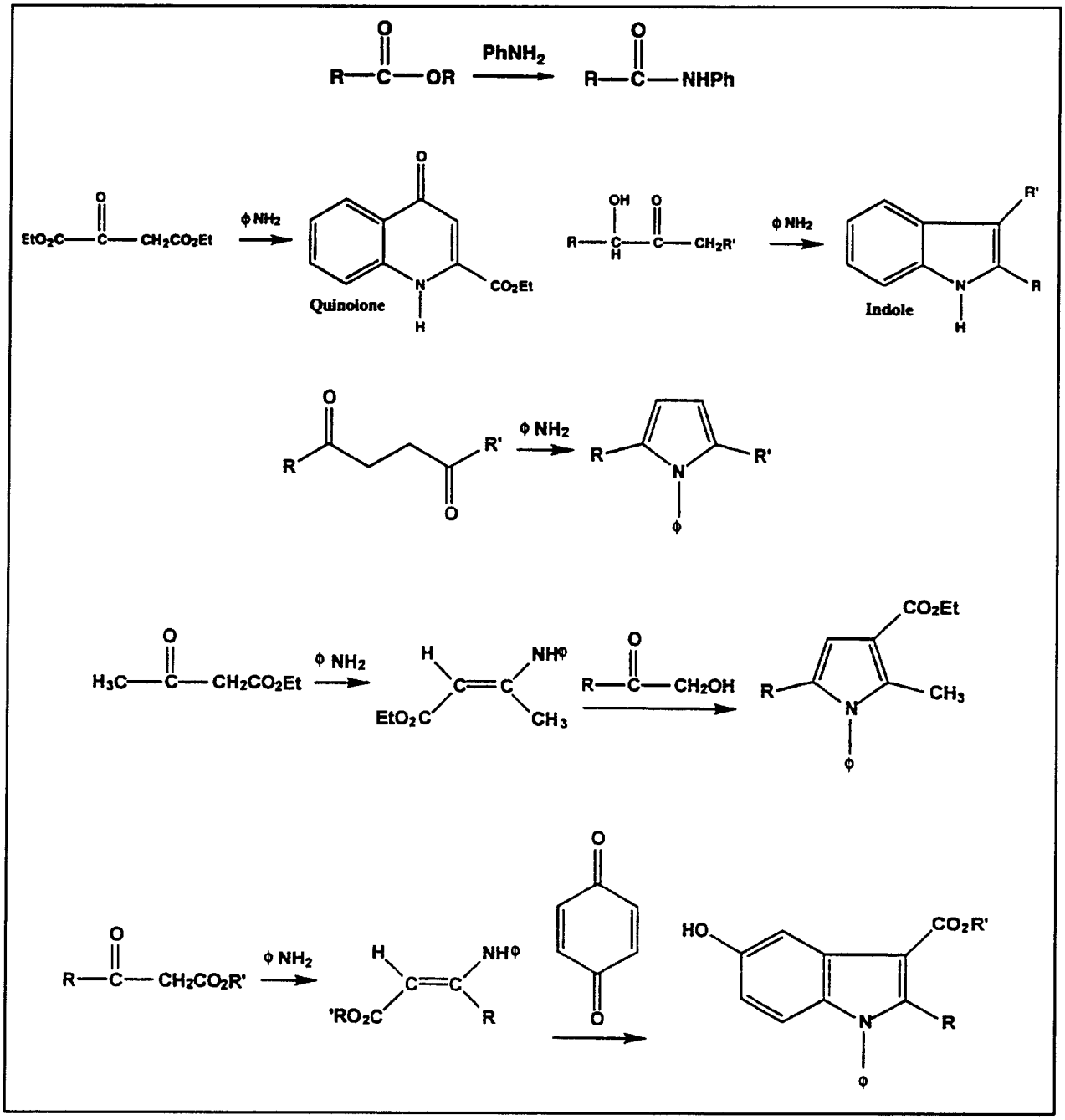


Figure 1. (Sheet 2 of 5)

Phenol Oxidase Enzymes (peroxidases, tyrosinases, laccases) and Metals (oxides or oxyhydroxides of Al, Fe, and Mn) Promote the One Electron Oxidation of Contaminant Aromatic Amines and of Aromatic Moieties within Humic Substances:

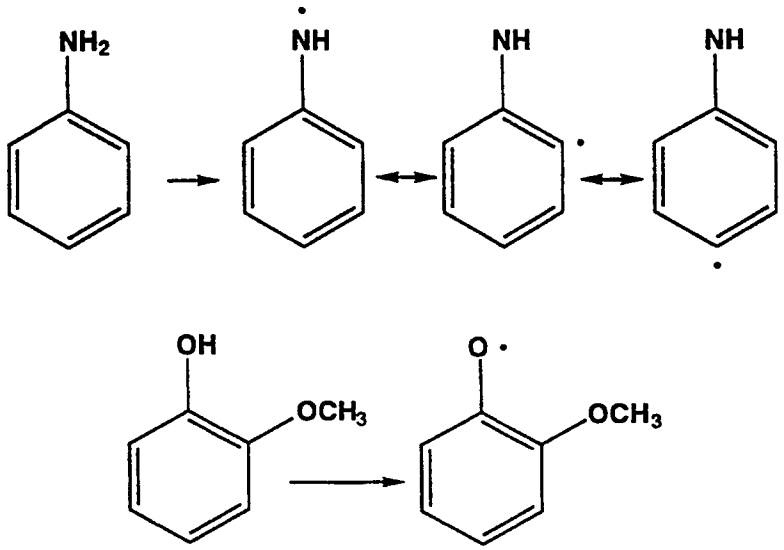


Figure 1. (Sheet 3 of 5)

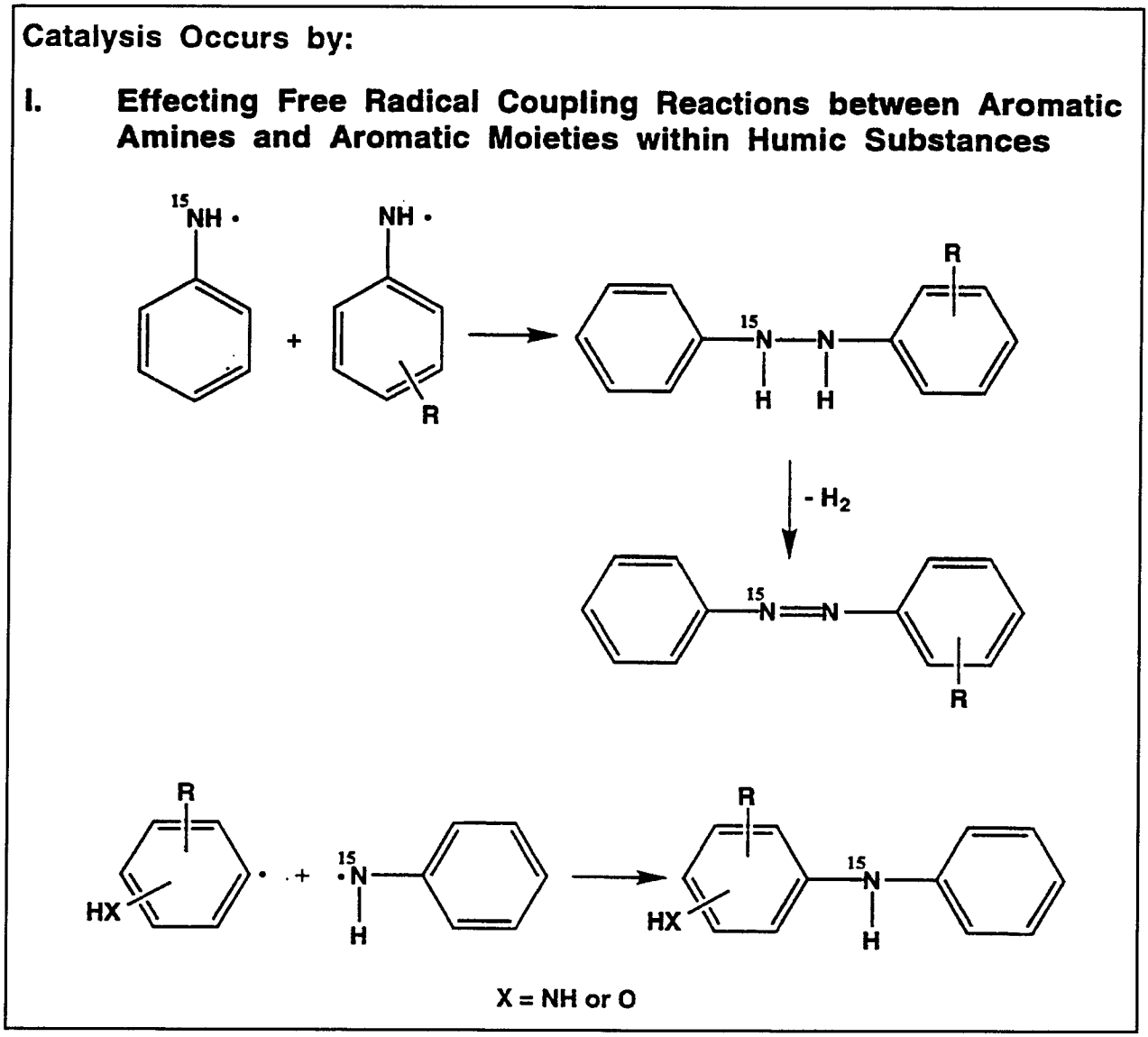


Figure 1. (Sheet 4 of 5)

II. Creating Additional Substrate Sites within Humic Molecules for Subsequent Nucleophilic Addition Reactions by Aromatic Amines

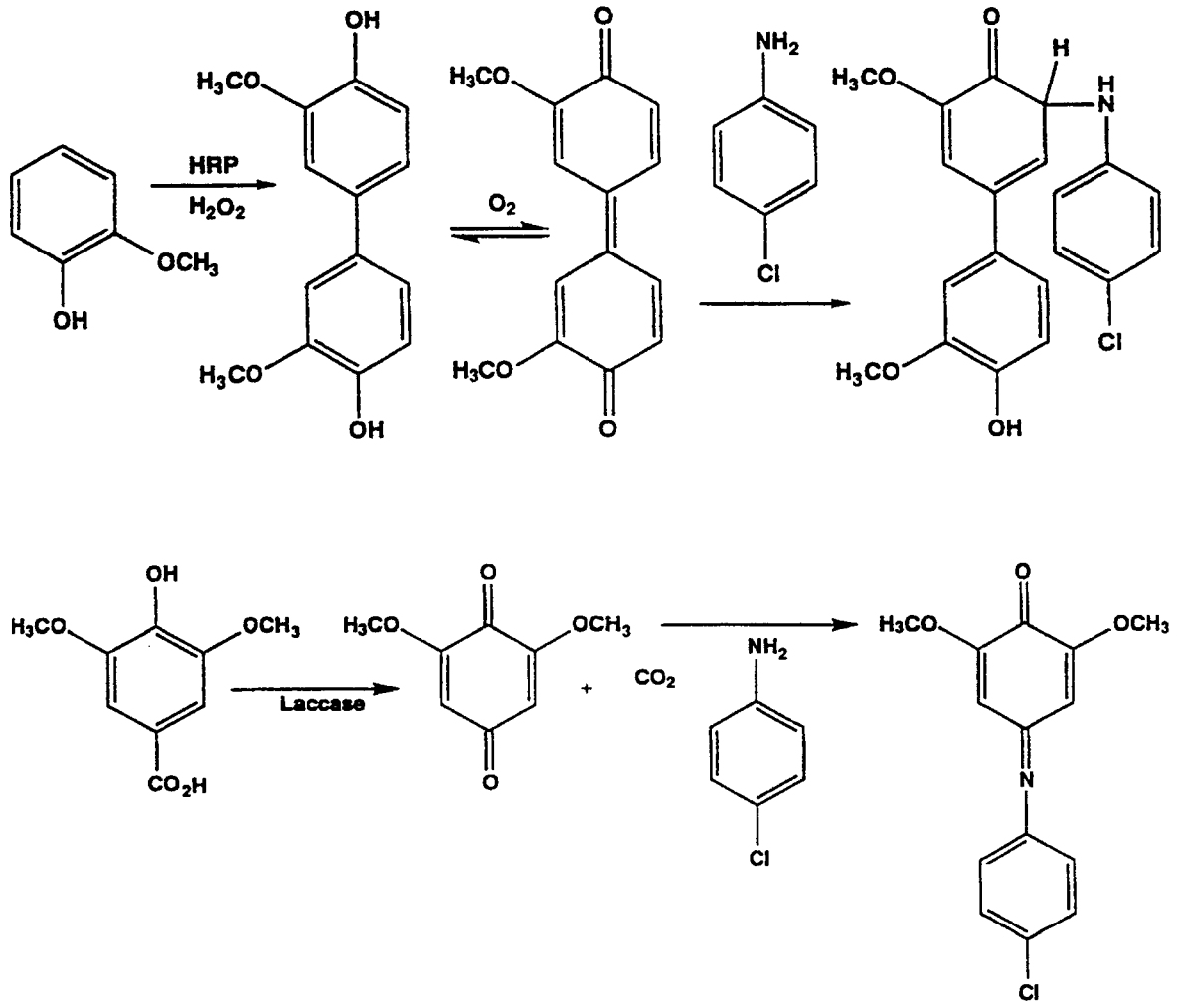


Figure 1. (Sheet 5 of 5)

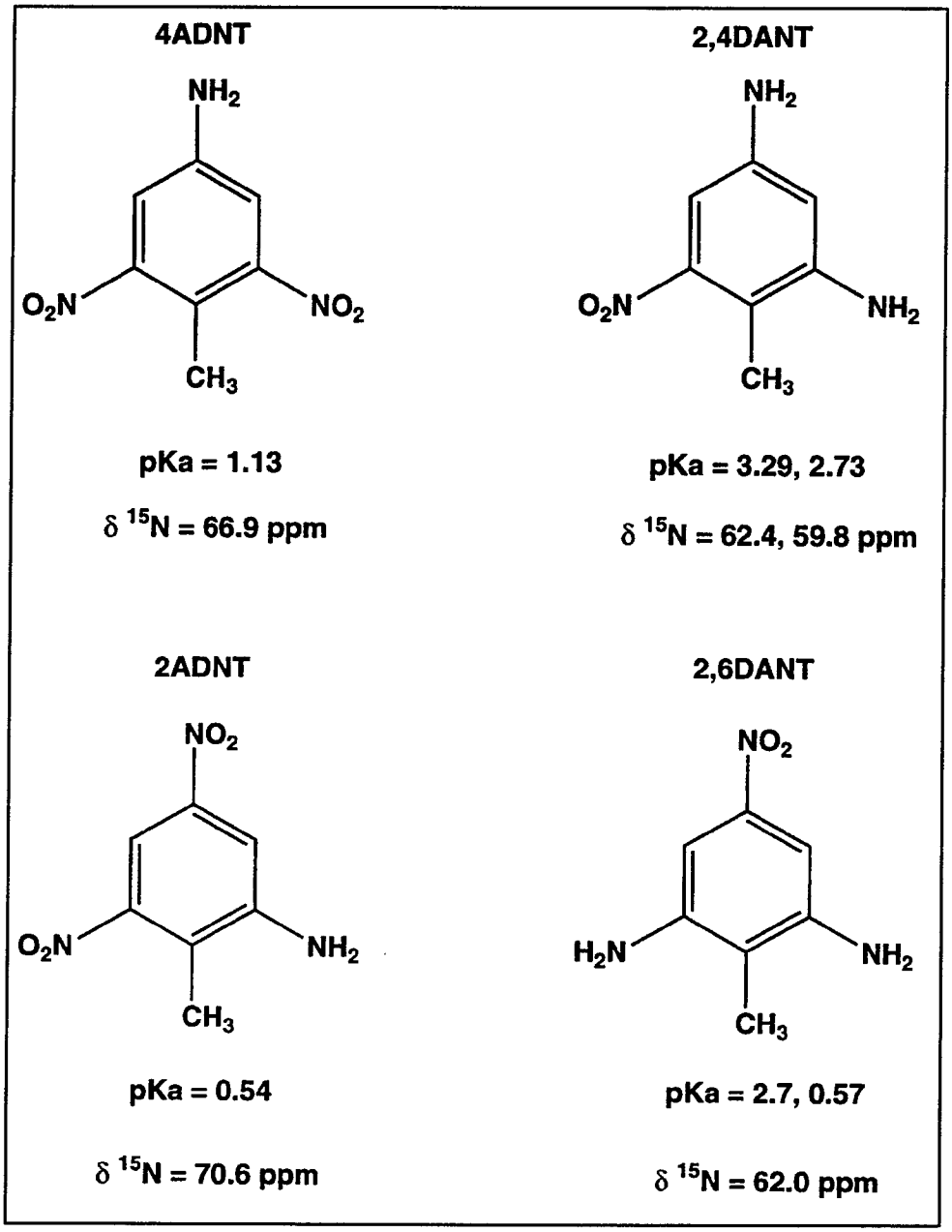


Figure 2. Liquid-phase d^{15}N NMR chemical shifts and pKa's of amines

The peaks from approximately 120 to 150 ppm are unidentified. The types of nitrogen that occur in this chemical shift range include amide, indole, and quinolone nitrogens. Only a few products result from the reaction of 2ADNT with 4MC, consistent with the lower pKa and presumed weaker nucleophilicity of 2ADNT compared with 4ADNT.

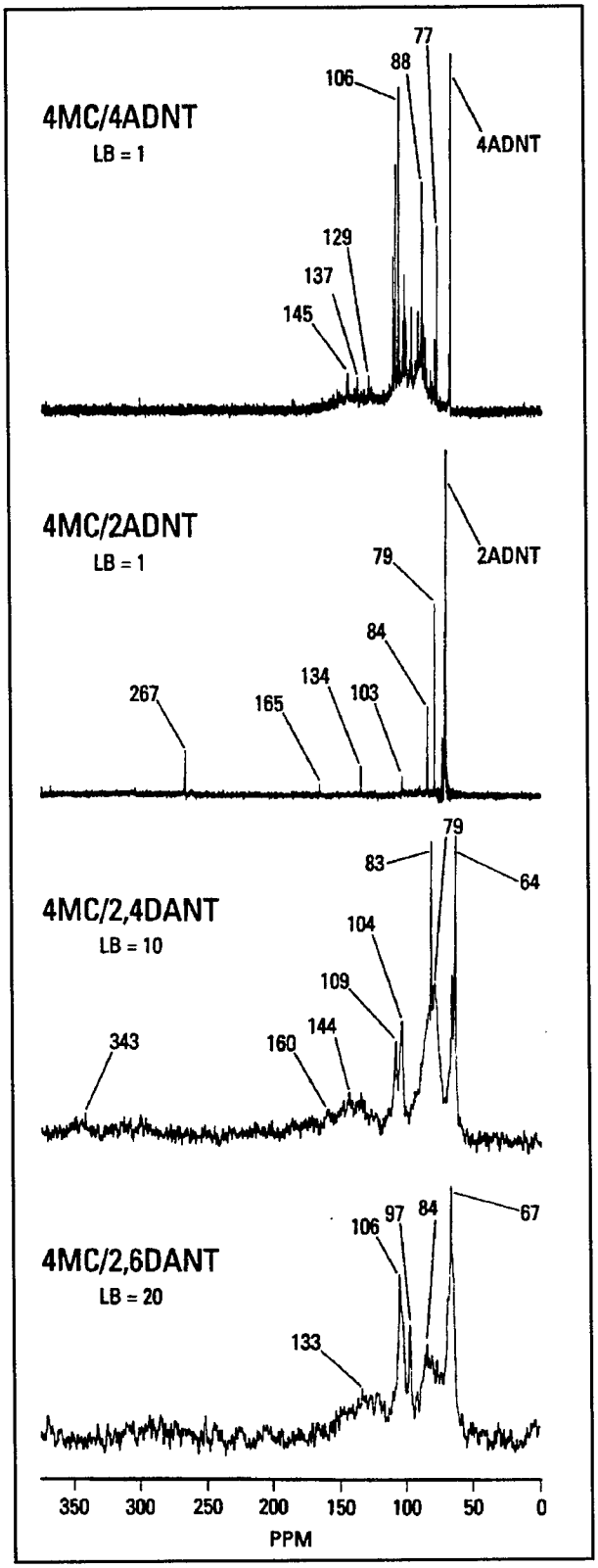
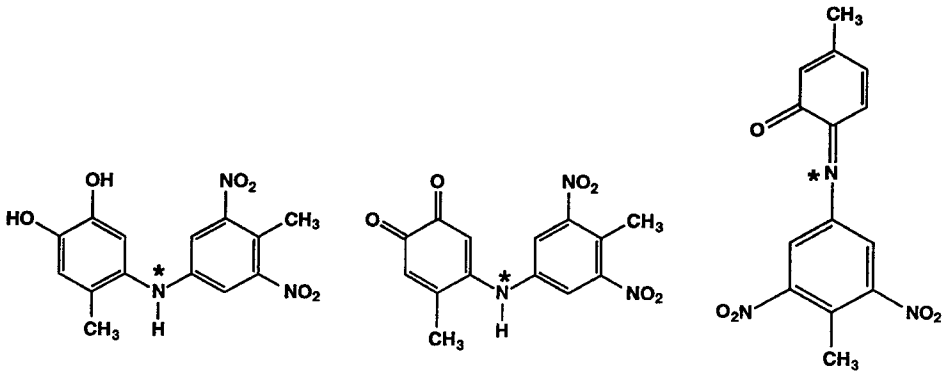


Figure 3. Liquid-phase inverse gated decoupled ^{15}N NMR spectra of 4-methylcatechol reacted with amines

The spectrum of the product mixture from the reaction of 2,4DANT and 4MC exhibits mainly broad bands, in contrast to the spectra of the monoamine reaction mixtures. The broad bands can be interpreted as representing a product mixture with a number of components much greater than in the monoamine mixtures. This again is consistent with the higher pKa and greater nucleophilicity of 2,4DANT. Determining directly from the spectrum which of the two amino groups has condensed with the quinone is not possible because both amine positions in the 2,4DANT are labeled. The 2-amino group is assumed to be less reactive than the 4-amino group because of steric hindrance effects. The broad peak at 65 ppm corresponds to the free amino groups (* in figures below) of the 2,4DANT molecules condensed with a 4MC molecule or larger oligomeric unit (trimer, tetramer, etc.).



Aminohydroquinone Aminoquinone Imine

Blank reactions of amines with peroxidase

The individual amines were incubated with peroxidase as controls for the peroxidase-catalyzed reactions of the amines with 4MC, conniferyl alcohol, and soil humic acid. The monoamines 4ADNT and 2ADNT did not undergo self-condensation reactions when reacted with peroxidase. No color changes occurred upon addition of hydrogen peroxide to the solutions of the enzyme and 4ADNT or 2ADNT. The solution of 2,4DANT darkened considerably upon addition of H_2O_2, whereas the solution of 2,6DANT darkened only slightly. A number of peaks are present in the spectrum of the 2,4DANT reaction blank (Figure 4) : 310, 292, and 269 ppm (azoxybenzene or imine nitrogen); 153, 134, 120, and 115 ppm (assignments uncertain); 82 and 79 ppm (hydrazine nitrogen). Only a few peaks are present in the spectrum of the 2,6DANT reaction blank, at 116 and 293 ppm.

Reaction of amines with conniferyl alcohol

The polymerization of conniferyl alcohol by peroxidase is considered one of the biosynthetic pathways to the formation of lignin in woody plant tissue. Studies on the phytoremediation of TNT have provided evidence that 4ADNT and 2ADNT form bound residues with plant tissue (Palazzo and Leggett 1986 ;

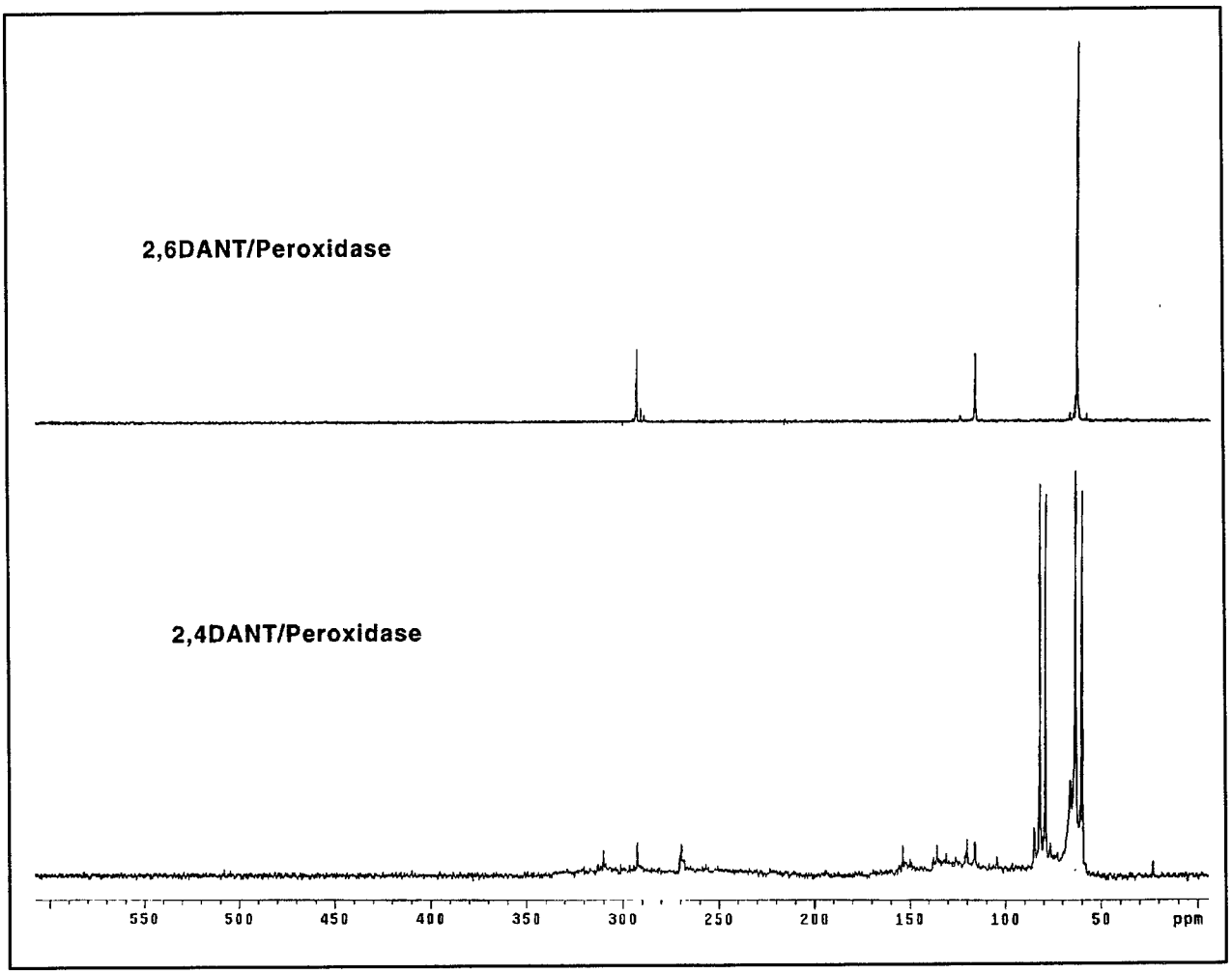


Figure 4. Liquid-phase inverse gated decoupled ^{15}N NMR spectra control reactions of 2,4DANT and 2,6DANT with horseradish peroxidase

of these bound residues can be derived from the work of von der Trenck, Hunkler, and Sandermann (1981), who studied the condensation of 4 -chloroaniline with conniferyl alcohol in the presence of peroxidase. Additionally, conniferyl alcohol moieties are plausible structural units within soil humic substances. The peroxidase-catalyzed reaction of 4ADNT and 2,4DANT with conniferyl alcohol was therefore examined as a model for the incorporation of the amines both into woody plant tissue and soil humic substances. The spectrum of the peroxidase-catalyzed reaction of 4ADNT with conniferyl alcohol exhibits a major peak at 80 ppm (Figure 5). This chemical shift is consistent with the 4ADNT analog of the reaction product of 4-chloroaniline with conniferyl alcohol reported by von der Trenck, Hunkler, and Sandermann (1981):

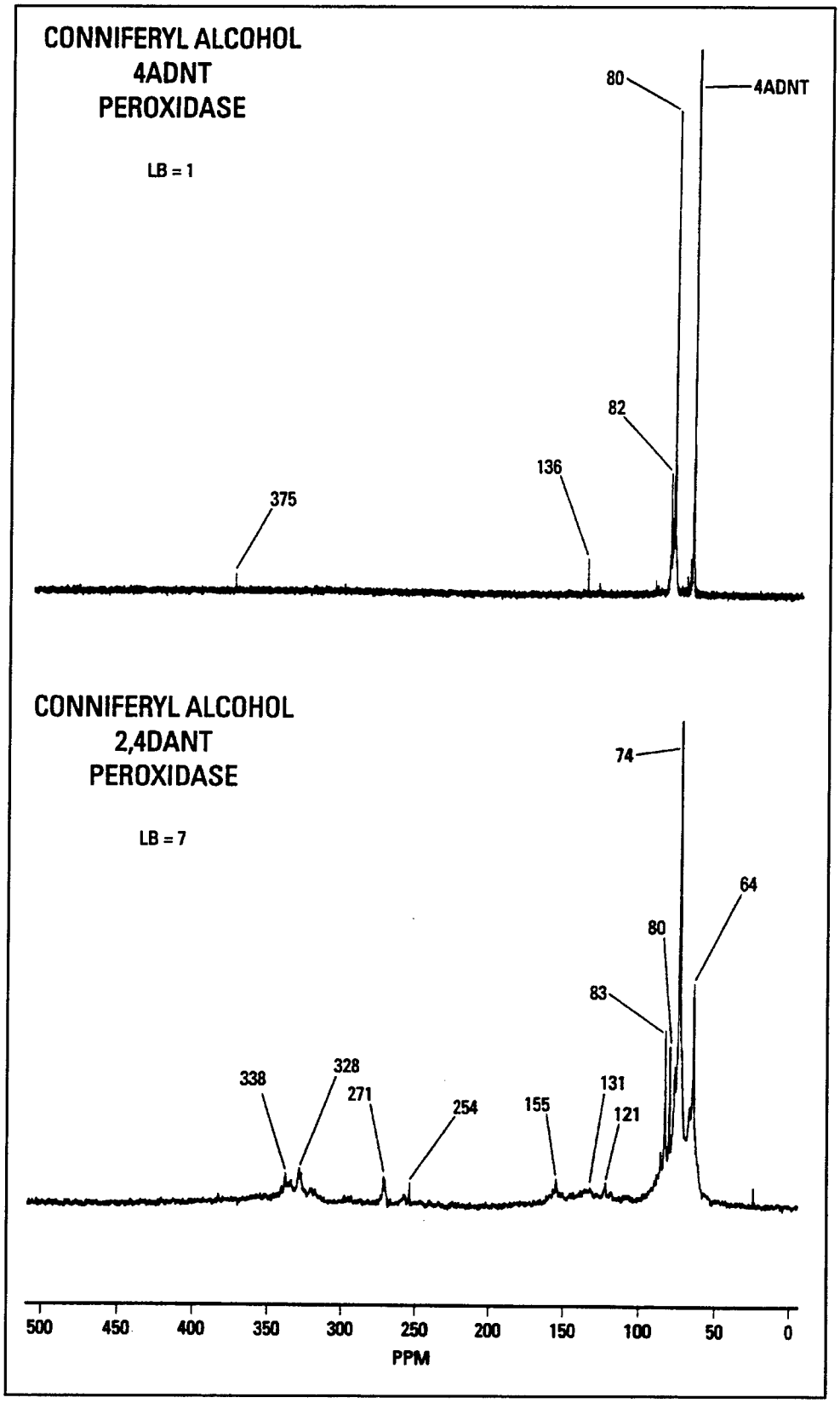
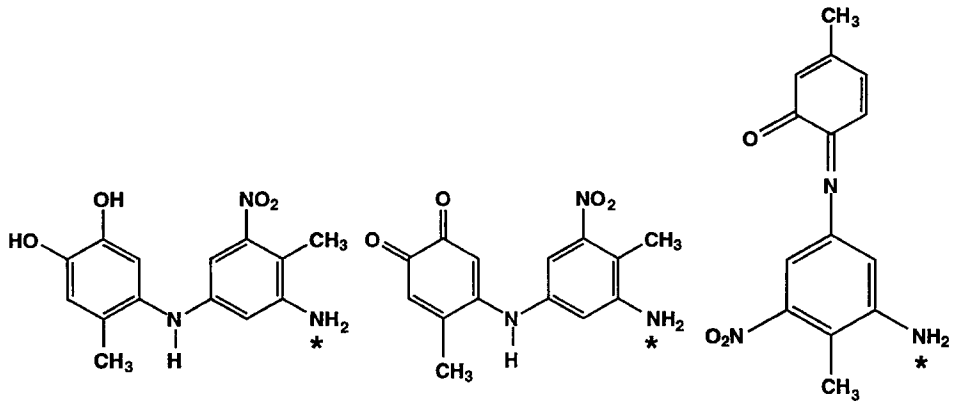


Figure 5. Liquid-phase inverse gated decoupled ^{15}N NMR spectra of peroxidase-catalyzed reactions of 4ADNT and 2,4DANT with conniferyl alcohol



Aminohydroquinone Aminoquinone Imine

The product mixture from the peroxidase-catalyzed reaction of conniferyl alcohol with 2,4DANT is complex. In addition to the major peak at 74 ppm, a broad band of resonances appears from approximately 100 to 160 ppm, peaks at 257 and 271 ppm, and imine nitrogens from 310 to 350 ppm.

Reaction of amines with soil humic acids

Both liquid-phase and solid-state CP/MAS ^{15}N NMR spectra were recorded on the soil humic acid samples reacted with the amines. Liquid-phase analyses provide quantitative accuracy and superior resolution.

The liquid-phase subspectral editing techniques are also more informative. However, the liquid-phase analyses require significantly more spectrometer time and in some cases become hampered by poor signal to noise when the amount of label incorporated into the samples is low. Faster analysis times and the ability to detect weak signals are the main advantages of solid-state CP/MAS NMR. A comparison between quantitative liquid-phase and solid-state CP/MAS spectra of the humic acids can be used to extrapolate the results of amines incorporation into whole soil or compost. The liquid-phase ^{15}N NMR analyses of the soil humic acids are discussed first.

Spectra of the soil humic acid reacted with 2,4DANT are shown in Figure 6. The ACOUSTIC spectra recorded with the addition of paramagnetic relaxation reagent (chromium III acetylacetonate) represent the quantitative distribution of nitrogens incorporated into the humic acid. The INEPT spectra show only nitrogens directly bonded to protons. In the ACOUSTIC spectrum of the soil humic acid from the nonenzyme reaction, the peak at 65 ppm corresponds primarily to the free amino groups of the 2,4DANT molecules bonded to the humic acid. Based upon assignments reported for humic substances reacted with aniline, the major bands in the ACOUSTIC spectrum can be interpreted as follows:

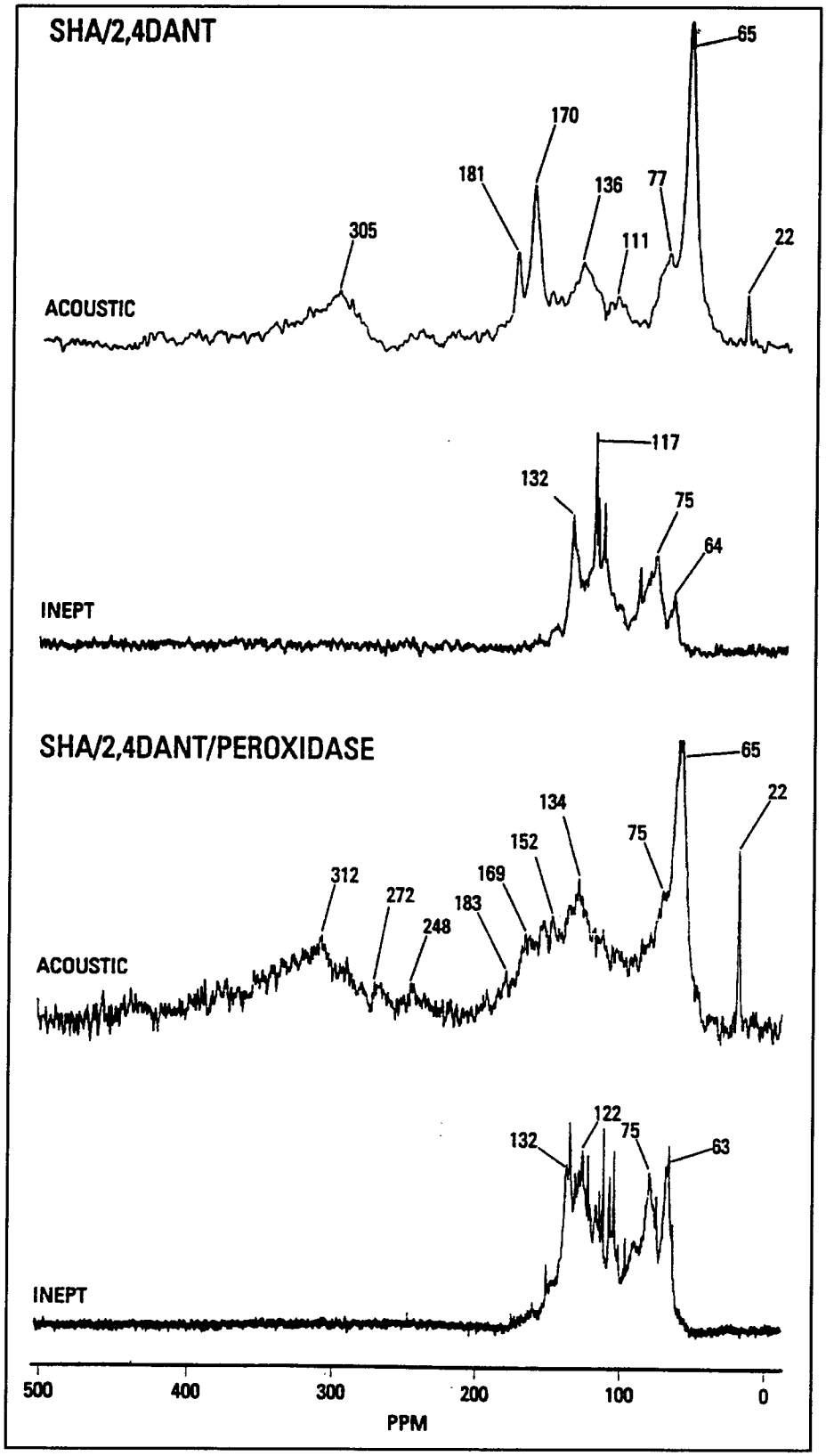


Figure 6. Liquid-phase ACOUSTIC and INEPT ^{15}N NMR spectra 2,4DANT reacted with IHSS soil humic acid

Chem Shift Range, ppm	Assignment
60 - 100	aminohydroquinone, phenoxazine
100 - 122	aminoquinone, enamine
122 - 148	amide, enaminone, quinolone, indole
148 - 200 heterocyclic N	N-phenylindole, N-phenylpyrrole,
300 - 350	imine, phenoxazinone, quinoline

The peak at 22 ppm corresponds to ammonia, which would result from deamination of 2,4DANT during the course of the condensation reactions. The INEPT spectrum indicates that resonances downfield of approximately 140 ppm in the ACOUSTIC spectrum represent nitrogens without protons directly attached, confirming that the peaks at 170 and 181 ppm in the ACOUSTIC spectrum correspond to heterocyclic nitrogens. Peak areas for the ACOUSTIC spectrum are listed in Table 1.

Table 1
Peak Areas as Percent of Total Nitrogen for ACOUSTIC ^{15}N NMR Spectra of Soil Humic Acid Reacted with Reduced TNT Amines in Presence and Absence of Peroxidase

	V	IV	III	II	I
Sample	350-275 ppm Imine	275-200 ppm Imidazole, Oxazole, Pyrazole, Nitrile	200-140 ppm Heterocyclic	140-70 ppm Amide, Aminoquinone, Amino-hydroquinone	70-50 ppm Nonbonded Amino
2,4DANT	15	0	26	28	30
2,4DANT/peroxidase	25	6	20	31	18
2,6DANT	16	0	26	35	23
2,6DANT/peroxidase	21	12	21	30	16
4ADNT	0	0	27	73	0
4ADNT/peroxidase	4	0	38	58	0

An obvious effect of peroxidase on the reaction of 2,4DANT with the humic acid is that the relative amount of imine formation is increased at the expense of heterocyclic nitrogen formation. The peaks at 170 and 181 ppm in the ACOUSTIC spectrum of the nonenzyme reaction are significantly diminished in the peroxidase spectrum. Imine nitrogen in the peroxidase spectrum comprises 25 percent of the nitrogen in the sample, compared with 15 percent in the nonenzyme reaction. The increase in imine formation as a result of catalysis by peroxidase was observed in the aniline studies. Peroxidase catalyzes the formation of 1,4-quinone groups from 3,5-disubstituted-4-hydroxybenzene carboxylic acid moieties (e.g., syringic acid) via an oxidative decarboxylation mechanism. The 1,2-addition of 2,4DANT to these hindered 1,4-quinone groups to form stable imine adducts is apparently favored over the sequence of condensation reactions resulting in the formation of heterocyclic nitrogen adducts. The very weak peaks

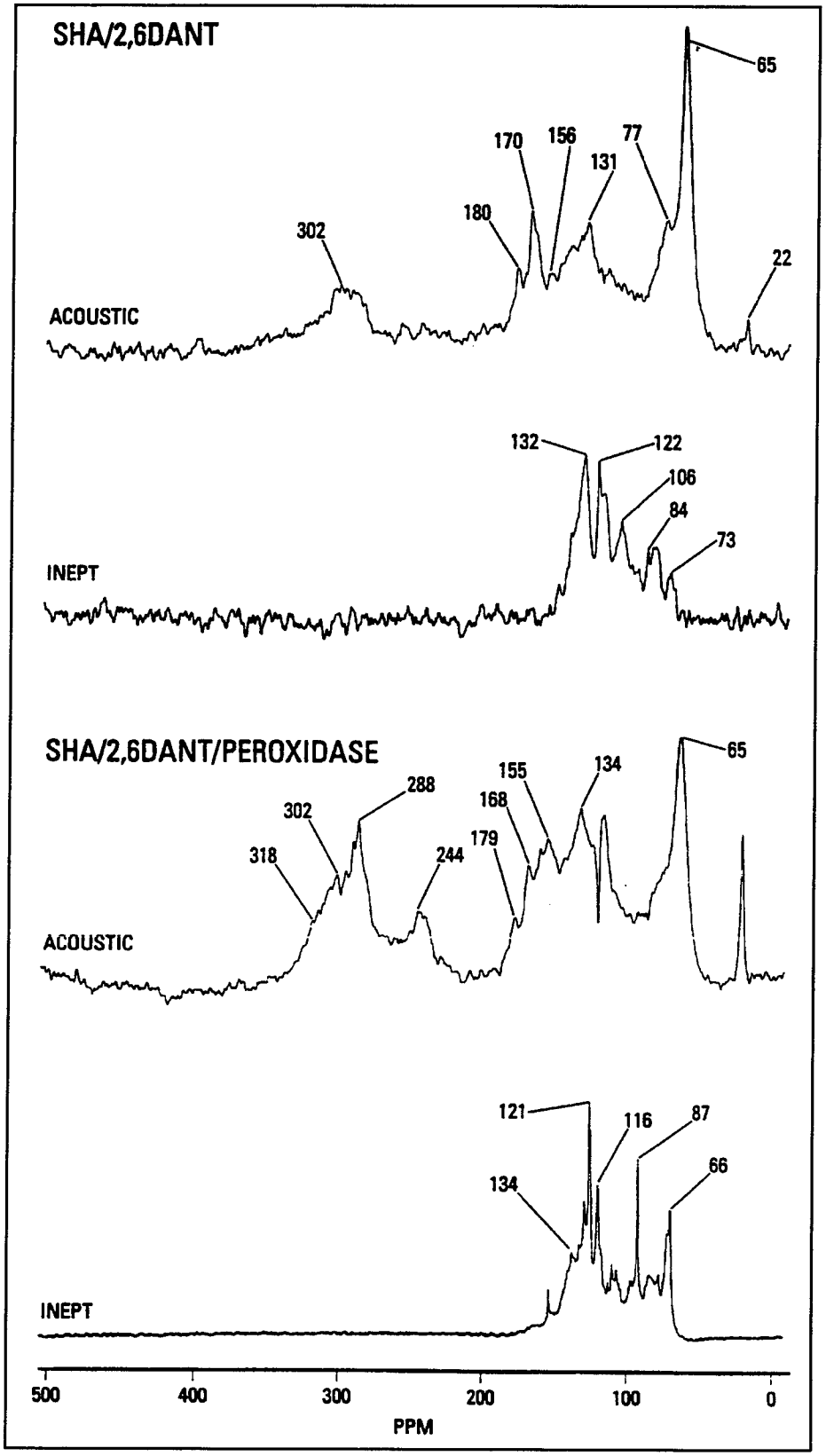


Figure 7. Liquid-phase ACOUSTIC and INEPT ^{15}N NMR spectra 2,6DANT reacted with IHSS soil humic acid

at 248 and 272 ppm may correspond to the unidentified nitrogens (imidazoles, oxazoles, pyrazoles, or nitriles) reported in the aniline study, and seen more clearly in the case of the peroxidase-catalyzed reaction of 2,6DANT with the humic acid (Figure 7). The intensity of the ammonia peak at 22 ppm suggests that more deamination occurs in the presence of the enzyme. A summary of assignments for both the nonenzyme and peroxidase-catalyzed reactions is listed in Table 2.

Table 2
Summary of Assignments for ^{15}N NMR Spectra of Samples Reacted with ^{15}N-Labeled Aniline

Noncatalyzed Reactions	
Chem Shift Range, ppm	**Assignment[1]**
60-100	*Anilinohydroquinone*, phenoxazine
100-122	*Anilinoquinone*, enamine
122-148	*Anilide*, enaminone, quinolone, indole
148-200	N-phenylindole, N-phenylpyrrole, *heterocyclic N*
300-350	*Imine*, phenoxazinone, quinoline
Peroxidase and Birnessite Catalyzed Reactions[2]	
60-100	Diphenylamine, hydrazine
230-280	Oxazole, imidazole, pyrazole, nitrile
310-360	*Iminodiphenoquinone, imine*, azoxybenzene
470-525	Azobenzene

[1] Most probable assignments indicated by italics.
[2] For samples reacted with aniline in the presence ofperoxidase and birnessite, assignments from both sections apply.

The nonenzyme reaction of 2,6DANT with the humic acid results in a distribution of condensation products similar to 2,4DANT, as evident from the close resemblance of the two sets of ACOUSTIC and INEPT spectra to one another (Figure 7). Aminohydroquinone, aminoquinone, amide, heterocyclic, and imine nitrogens are all present. The effects of peroxidase on the reaction of 2,4DANT with the humic acid are also replicated in the case of 2,6DANT. Imine formation is increased at the expense of heterocyclic nitrogen formation. The unidentified nitrogens also are unequivocally present at 244 ppm. Peroxidase also increased the release of ammonia via deamination.

The 4ADNT is less reactive towards the soil humic acid than the diamines, as indicated by the much weaker signal to noise of the ^{15}N NMR spectra (Figure 8). In the ACOUSTIC spectrum of the nonenzyme reaction, resonances occur from approximately 67 to 175 ppm, the resonances downfield of approximately 140 ppm comprising heterocyclic nitrogens. The very weak signal at 305 ppm

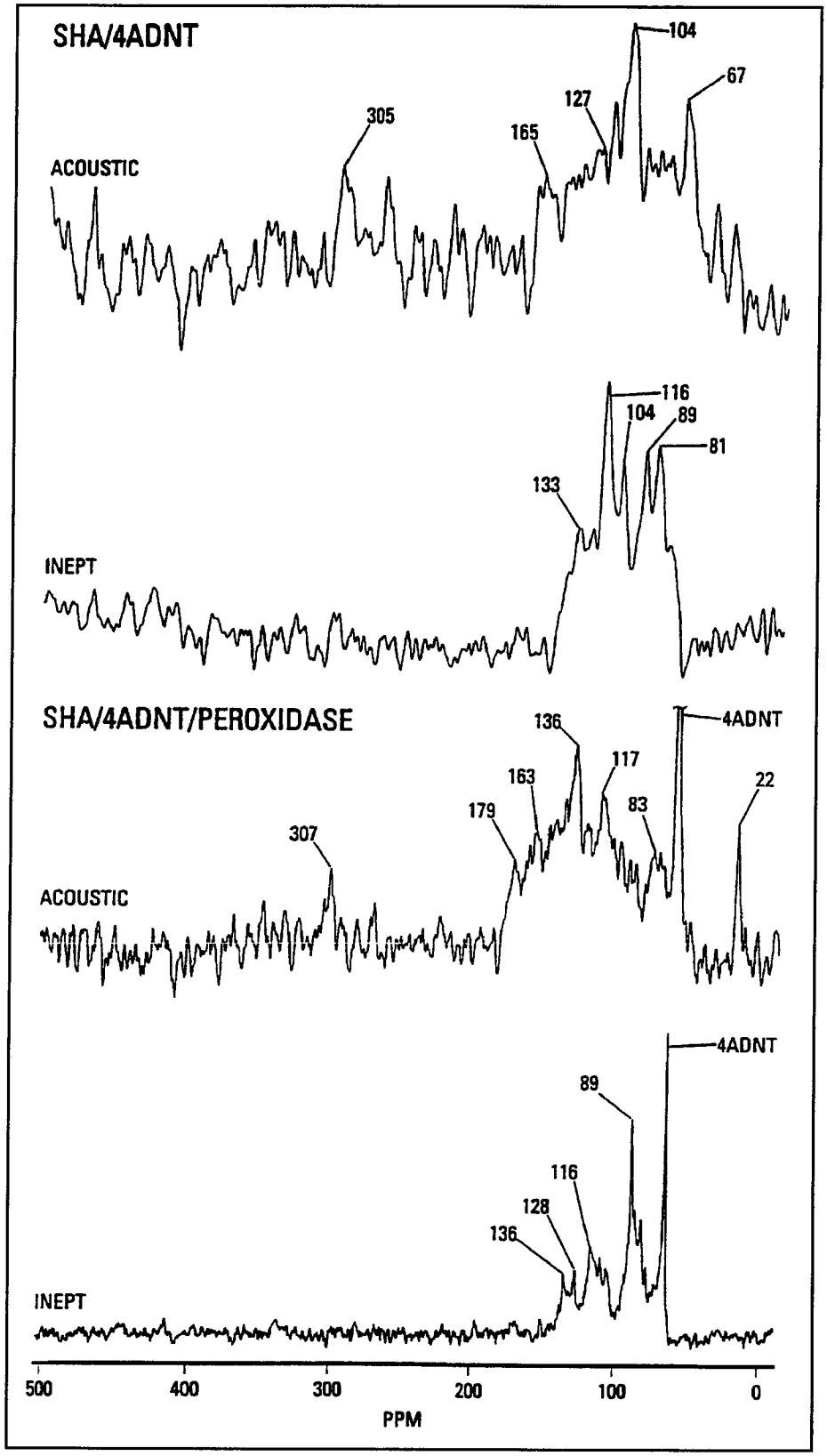


Figure 8. Liquid-phase ACOUSTIC and INEPT ^{15}N NMR spectra 4ADNT reacted with IHSS soil humic acid

may be an analytical artifact. In general, however, the lack of imine formation (1,2-addition of amine to quinones) distinguishes the reactivity of the monoamines from the diamines. The ACOUSTIC spectrum of the peroxidase-catalyzed reaction of 4ADNT with the humic acid shows improved signal to noise over the spectrum of the nonenzyme reaction, indicating that peroxidase increases the incorporation of the amine into the humic acid. The region of heterocyclic nitrogens (140 to 185 ppm) is more clearly defined in this spectrum, important because these nitrogens are not as clearly resolved in the corresponding CP/MAS spectrum (Figure 9). The peak at 307 ppm exhibited a poor signal-to-noise ratio and may not be real. An ammonia peak is again observed at 22 ppm.

The signal-to-noise ratios of the liquid-phase ^{15}N NMR spectra of the soil humic acid reacted with 2ADNT were too weak to report, so attention will be focused on the solid-state spectra (Figures 9-11). The CP/MAS spectra of the humic acid reacted with 2ADNT and 4ADNT resemble one another fairly closely. Despite its lower reactivity toward 4-methylcatechol compared with 4ADNT, 2ADNT appears to react with the humic acid similarly to 4ADNT in forming aminohydroquinone, aminoquinone, amide, and heterocyclic nitrogen adducts. 2ADNT forms the same condensation products in the presence of peroxidase, but with the possible addition of some imine nitrogen at 338 ppm.

Hydrolysability of covalent bonds between amines and peat

Solid-state CP/MAS spectra before and after hydrolysis of the Pahokee peat reacted with aniline are shown in Figure 12. This spectrum most clearly illustrates the types of bonds susceptible to hydrolysis. Clearly the peaks at 76, 99, and 332 ppm are significantly diminished after hydrolysis, indicating that aniline bonded to the peat via aminohydroquinone, aminoquinone, and imine linkages is released. The chemical shift position of the peak at 133 ppm is shifted downfield to 135 ppm after hydrolysis, indicating some loss of amide nitrogen as well. Aniline bonded to the peat in the form of heterocyclic condensation linkages is stable to hydrolysis.

Somewhat similar results are obtained with the peat samples reacted with the reduced TNT amines, although the spectra (Figures 13-16) are not as clearly resolved as in the case of aniline. The unreacted amines were not completely removed from the peat during dialysis. In the spectra before hydrolysis, peaks due to the unreacted amines overlap with the resonances due to aminohydroquinone and to some extent aminoquinone bonds. In the case of 2,4DANT, peak maxima in the spectra of the unhydrolyzed sample occur at 56, 120, and 328 ppm (Figure 13). The chemical shift position of the peak at 120 ppm moves downfield to 140 ppm after hydrolysis, indicating the loss of 2,4DANT bonded to the peat via aminoquinone and amide linkages. The 2,4DANT bonded to the peat via imine linkages is also released upon hydrolysis. Some amines bonded to the peat via aminohydroquinone bonds persist after hydrolysis (peak at 72 ppm). The remaining peaks at 57 and 46 ppm in the hydrolyzed sample correspond to the free amino groups of the 2,4DANT molecules still bonded to the peat. These observations are

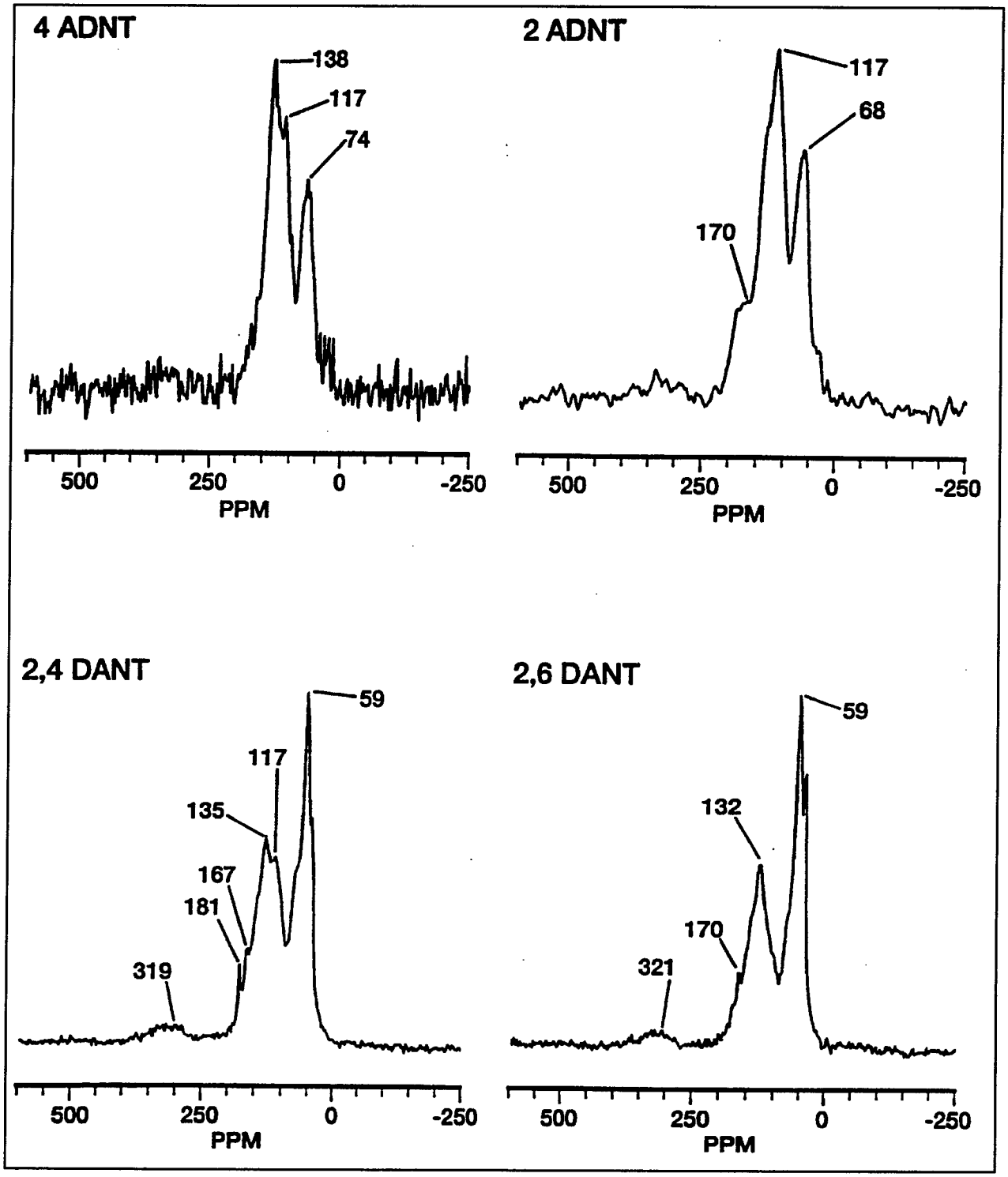


Figure 9. Solid-state CP/MAS ^{15}N NMR spectra of amines reacted with soil humic acid (no catalyst)

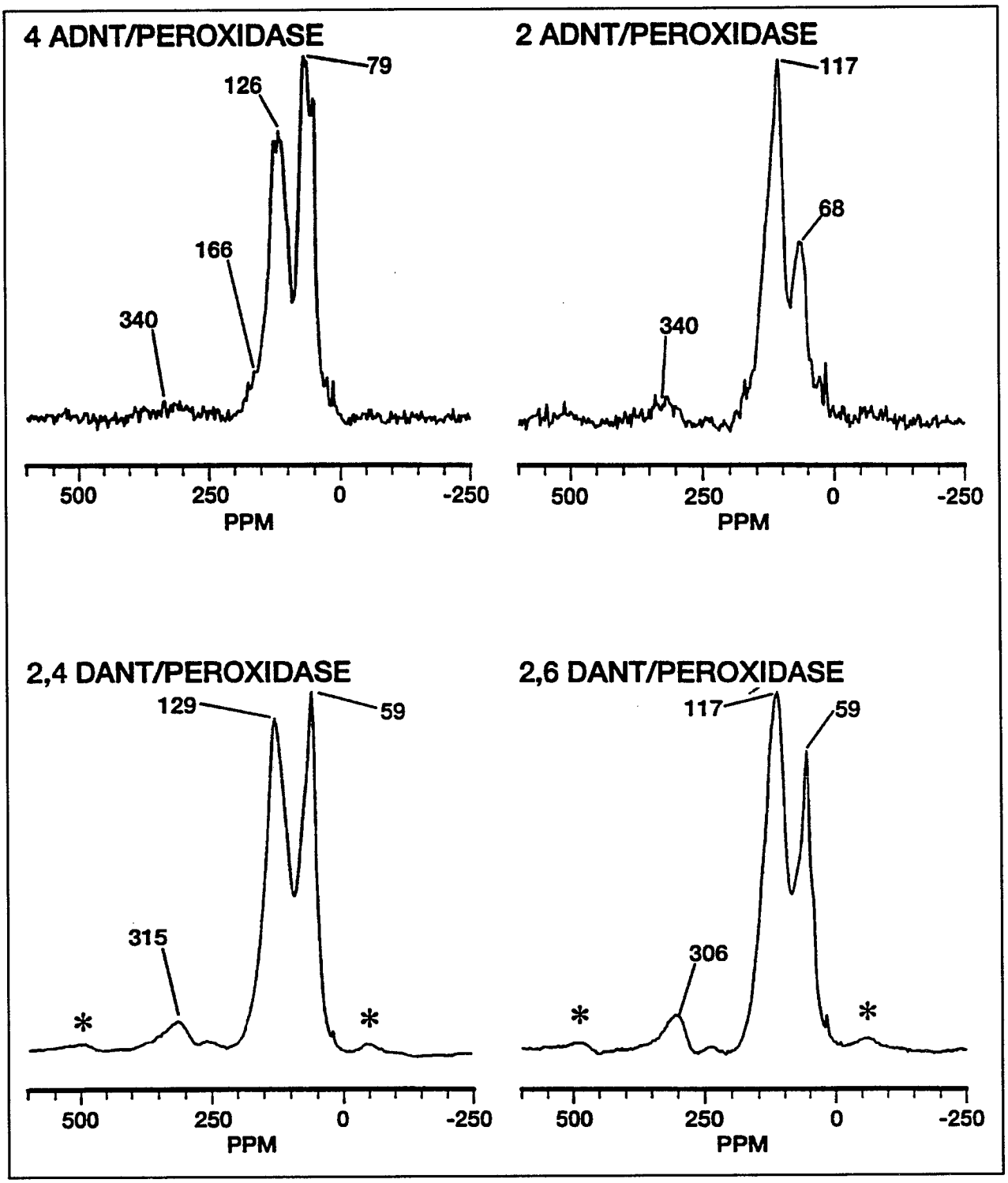


Figure 10. Solid-state CP/MAS ^{15}N NMR spectra of amines reacted with soil humic acid (peroxidase catalyzed)

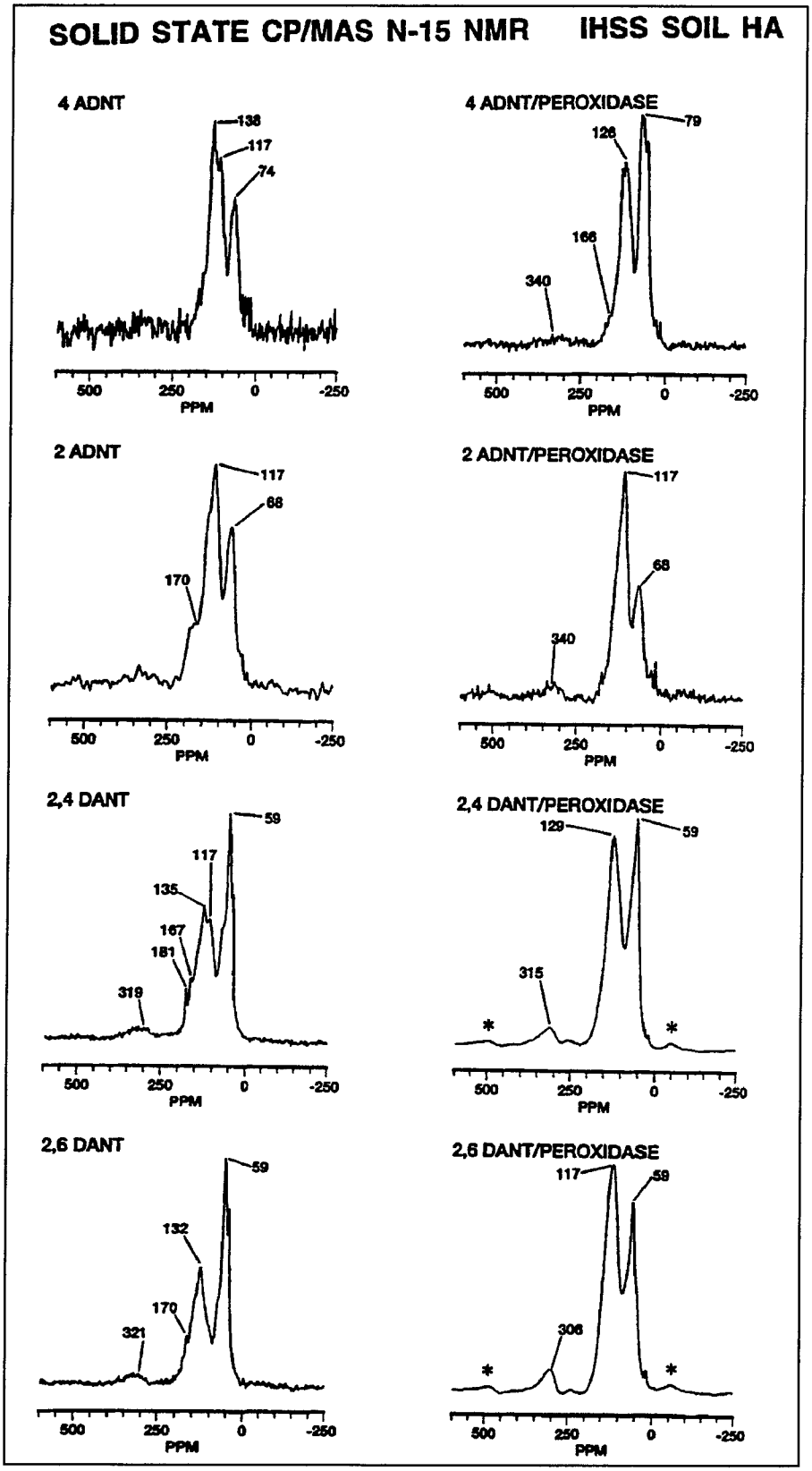


Figure 11. Solid-state CP/MAS ^{15}N NMR spectra of amines reacted with soil humic acid

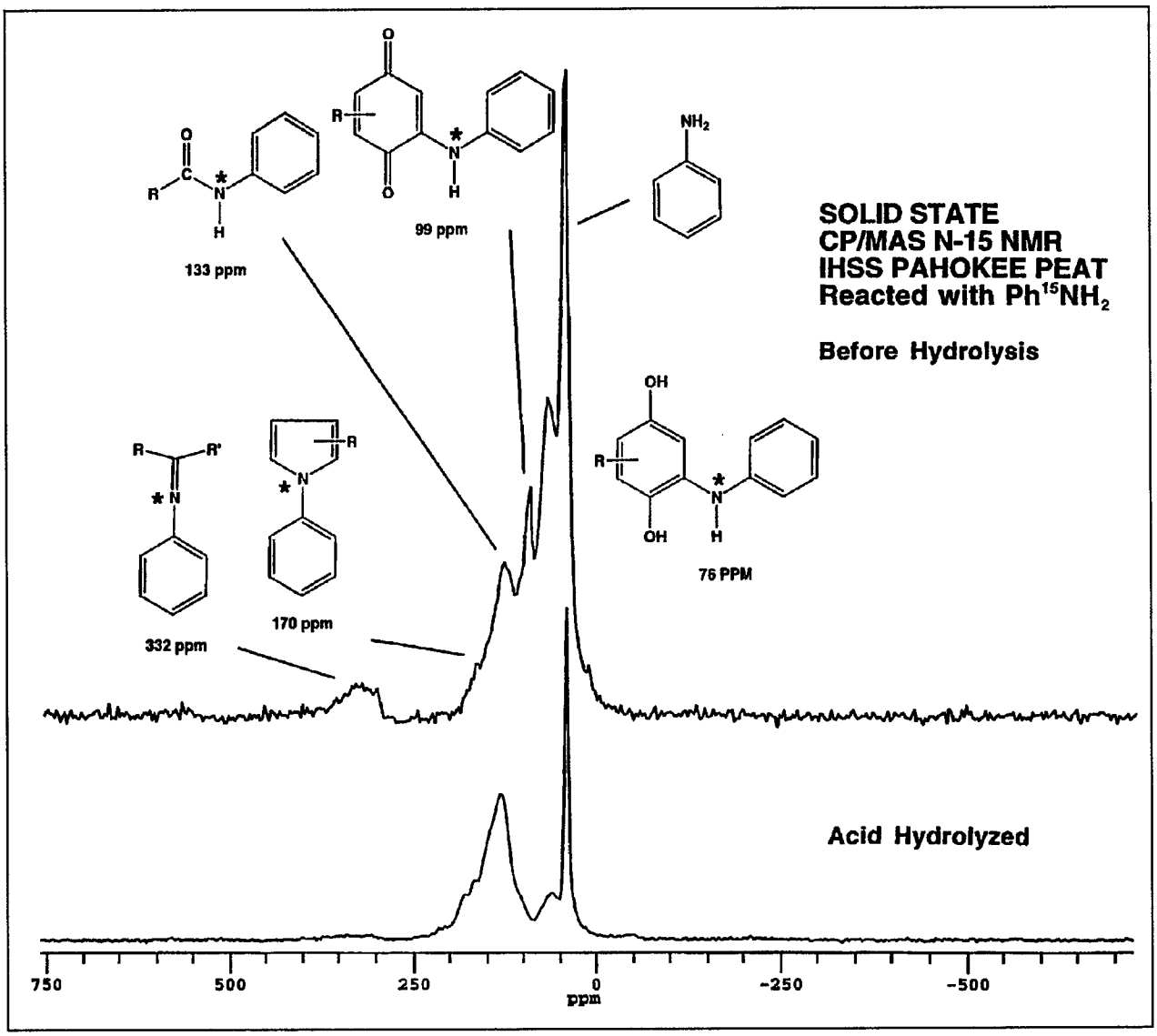


Figure 12. Solid-state CP/MAS ^{15}N NMR spectra of aniline-reacted Pahokee peat before and after hydrolysis

replicated in the case of 2,6DANT. One difference is that 2,6DANT reacts to form more imine nitrogen with the peat than 2,4DANT.

The monoamines 4ADNT and 2ADNT do not appear to undergo 1,2 -addition with carbonyl groups in the peat to form imines. The spectra of the hydrolyzed samples indicate that the heterocyclic adducts persist the hydrolysis.

Conclusions

The ^{15}N NMR studies have confirmed that the monoamines 2ADNT and 4ADNT and the diamines 2,4DANT and 2,6DANT undergo covalent binding to

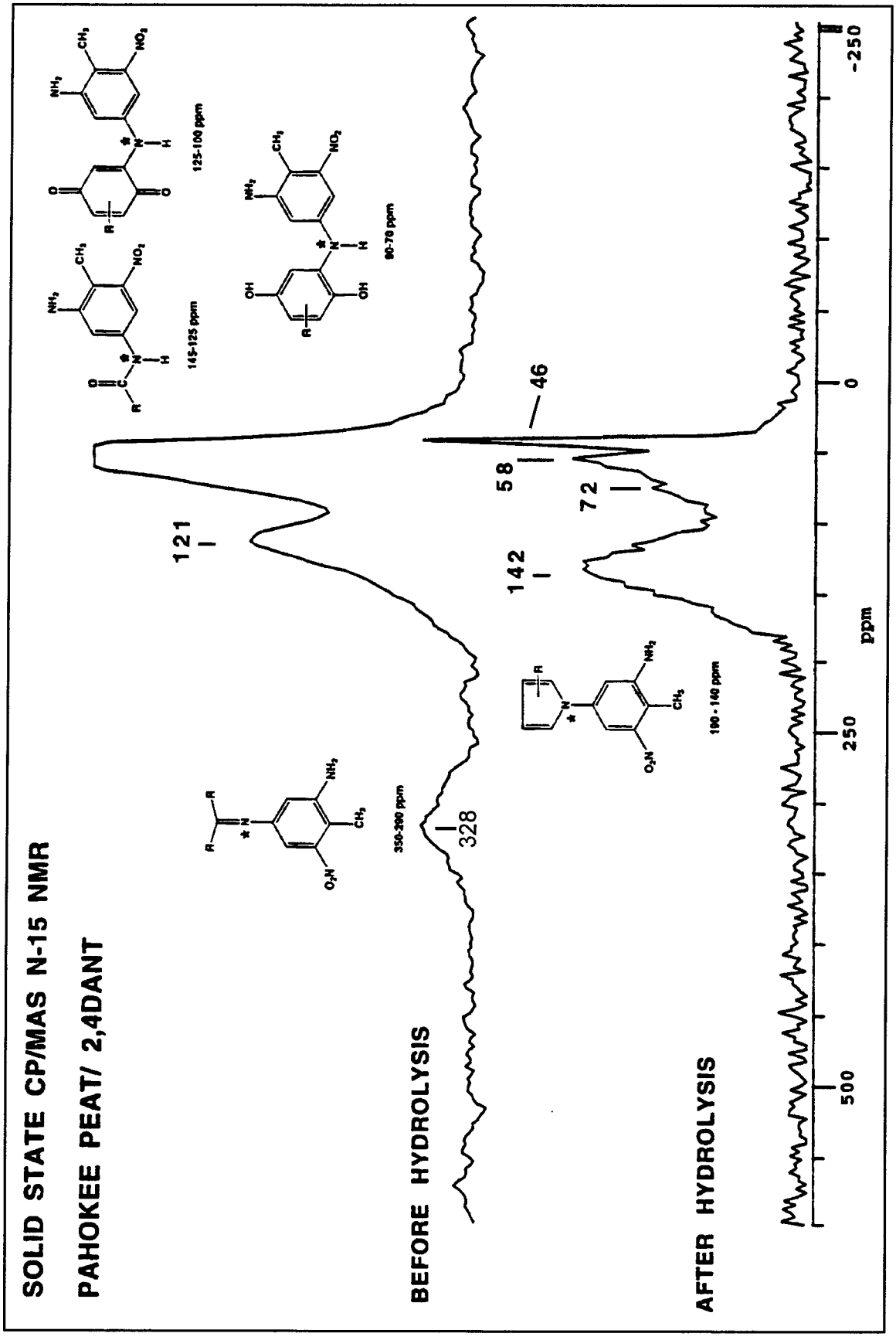


Figure 13. Solid-state CP/MAS ^{15}N NMR spectra of 2,4DANT-reacted Pahokee peat before and after hydrolysis

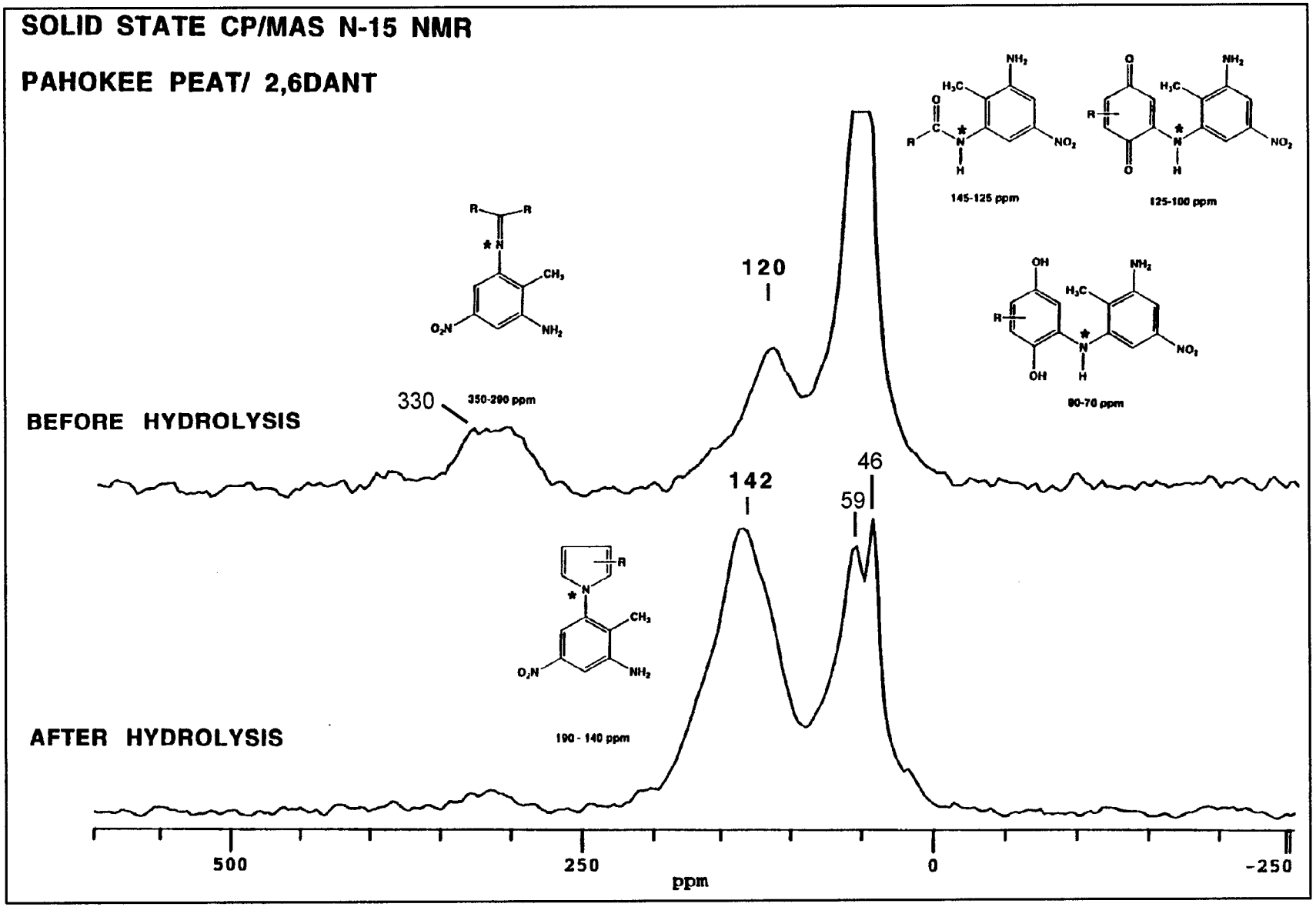


Figure 14. Solid-state CP/MAS ^{15}N NMR spectra of 2,6DANT-reacted Pahokee peat before and after hydrolysis

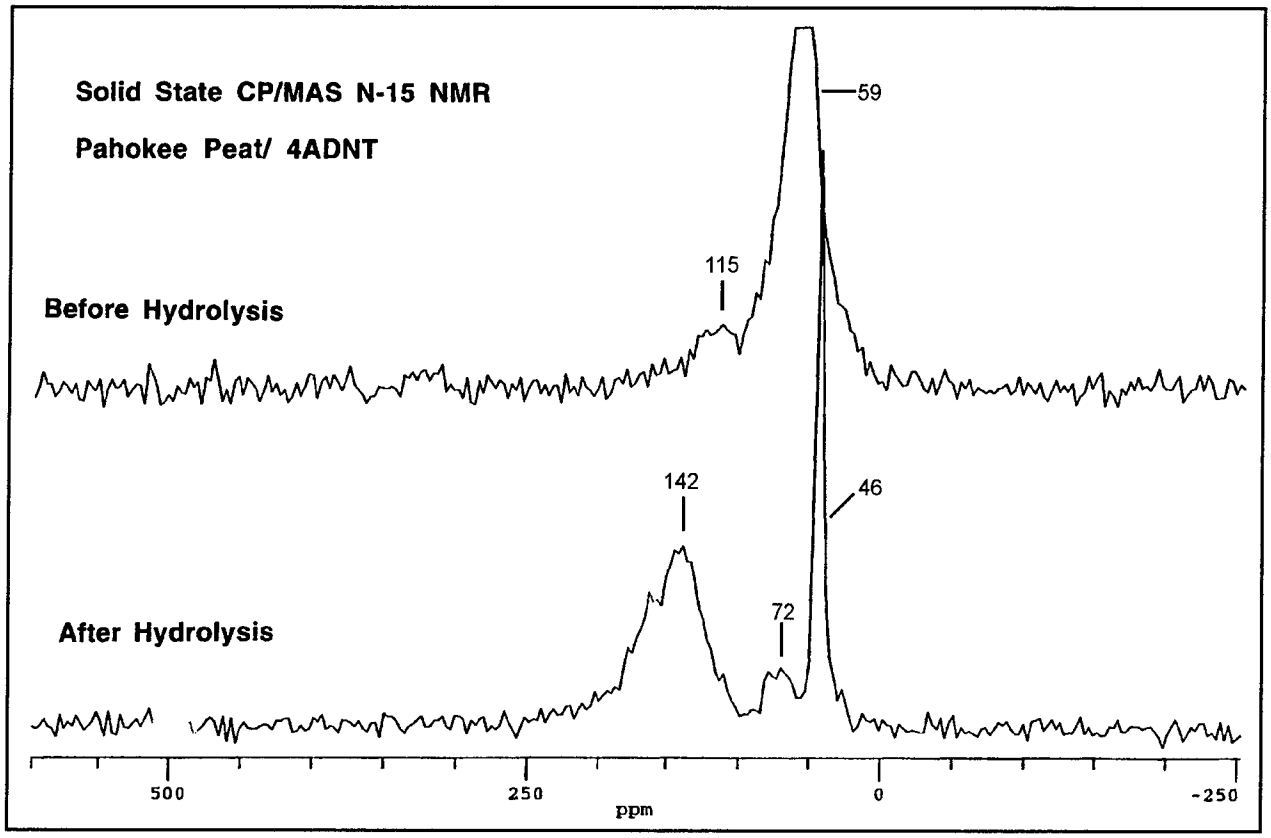


Figure 15. Solid-state CP/MAS ^{15}N NMR spectra of 4ADNT-reacted Pahokee peat before and after hydrolysis

soil humic acid, the organic matter of whole peat, and model quinone and lignin compounds. The confirmation of covalent binding explains how the metabolites of TNT form nonextractable-bound residues with organic matter in soil and compost. In the absence of catalysts, the amine groups of 2ADNT and 4ADNT undergo nucleophilic addition reactions with the carbonyl functionality of soil humic acid and peat to form aminohydroquinone, aminoquinone, amide, and heterocyclic adducts. The diamines 2,4DANT and 2,6DANT are more reactive than the mono amines, form the same types of adducts as the monoamines, but also become incorporated into the soil humic acid and peat via imine bonds. The phenol oxidase enzyme horseradish peroxidase increases the incorporation of all four amines into the humic acid. In the case of the diamines, the enzyme effects an increase in the amount of imine bond formation at the expense of heterocyclic bond formation. The manganese dioxide mineral birnessite also appears to have the same catalytic effects as horseradish peroxidase. Hydrolysis experiments on peat reacted with the individual amines indicate the following lability of the various types of bonds formed: amines bonded via heterocyclic condensation linkages are resistant to a combination of base and acid hydrolysis; amines bonded via aminohydroquinone, aminoquinone, and amide linkages are partially released upon base and acid hydrolysis; diamines bonded via imine linkages are almost completely released from base and acid hydrolysis.

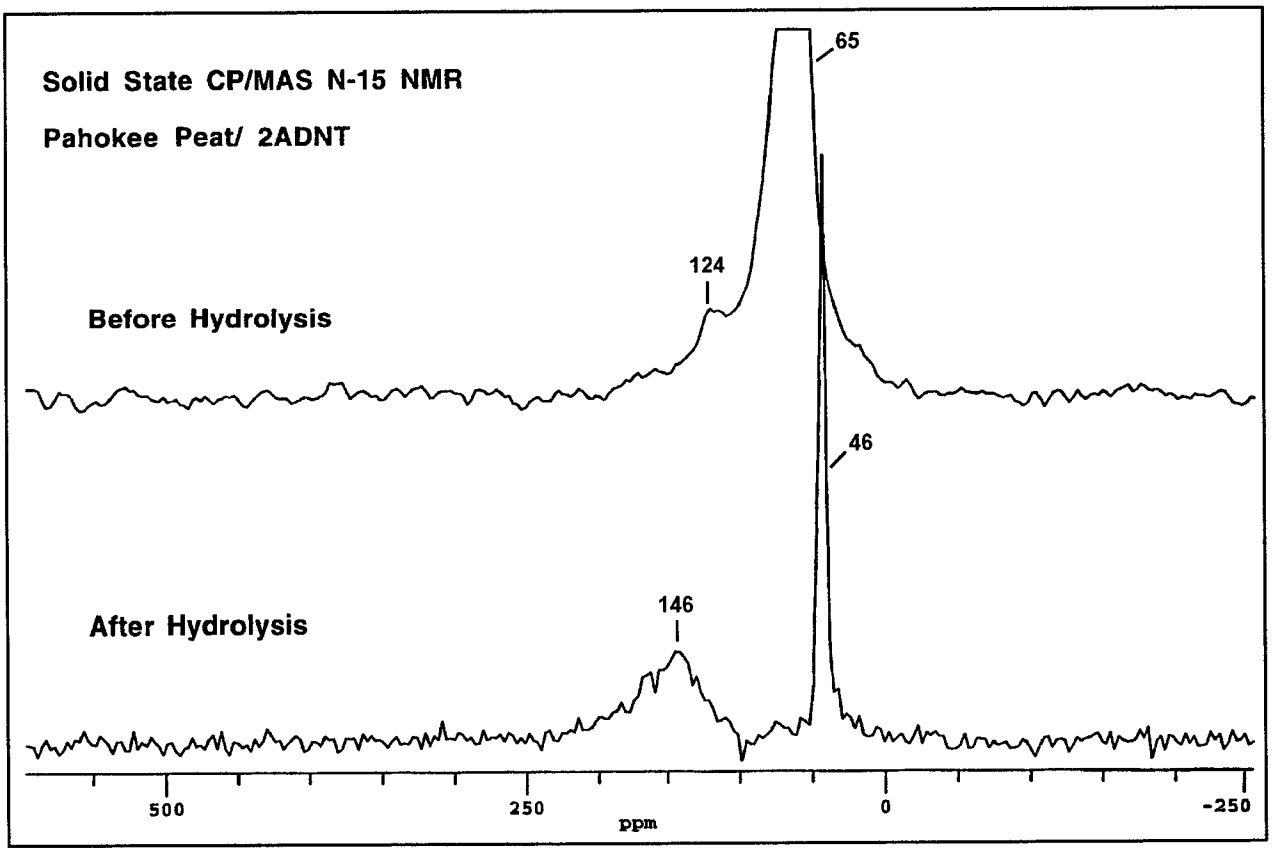


Figure 16. Solid-state CP/MAS ^{15}N NMR spectra of 2ADNT-reacted Pahokee peat before and after hydrolysis

At this stage of research, it is not known what role phenol oxidase enzyme and metal catalysts play in covalent bond formation during composting or under conditions of natural attenuation in soils. Future experiments in which ^{15}N NMR is used to follow the reduction and subsequent binding of ^{15}N-labeled TNT in whole compost systems may answer this question.

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3 Hydrolytic Release of Bound TNT Transformation Products from Composted Soil and Digester Sludge

Introduction

Previous work on biotreatment of TNT-contaminated soils has indicated that TNT is rapidly converted to solvent-extractable transformation products and to residues that are conjugated in a nonsolvent-extractable form (Kaplan and Kaplan 1982; Caton et al. 1994; Pennington et al. 1995). Plants grown hydroponically in TNT solutions metabolized TNT reductively, and transformation residues were partially conjugated in a nonsolvent-extractable form (Palazzo and Leggett 1986a,b; Harvey et al. 1990). A considerable quantity of the two aminodinitrotoluene (ADNT) transformation products were released by acid hydrolysis of the harvested plants after first extracting unconjugated transformation products with benzene (Palazzo and Leggett 1986a,b).

In experiments at Oak Ridge National Laboratory, base hydrolysis following solvent extraction recovered an additional 60 percent of the applied radiolabel from TNT/soil composts after 90 days (Caton et al. 1994). Although the hydrolysates were not analyzed for specific TNT transformation products, the analogy between living plants and remediation systems containing mostly plant matter and wastes suggested that standard HPLC analysis of hydrolysates could be used to identify and quantify conjugated TNT residues in compost and digester sludge.

TNT transformation and disposition in the environment have been thoroughly discussed in several reviews (McCormick, Feeherry, and Levinson 1976; Walsh 1990; Gorontzy et al. 1994). The biological reduction of the nitro groups on TNT proceeds through nitroso and hydroxylamine intermediates to two aminodinitrotoluene isomers (ADNTs), 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene (2ADNT), and two diaminonitrotoluene isomers (DANTs), 2,4-diamino-6-nitrotoluene (2,4DANT) and 2,6-diamino-4-nitrotoluene (2,6DANT). The condensation of hydroxylamine intermediates to form azoxy

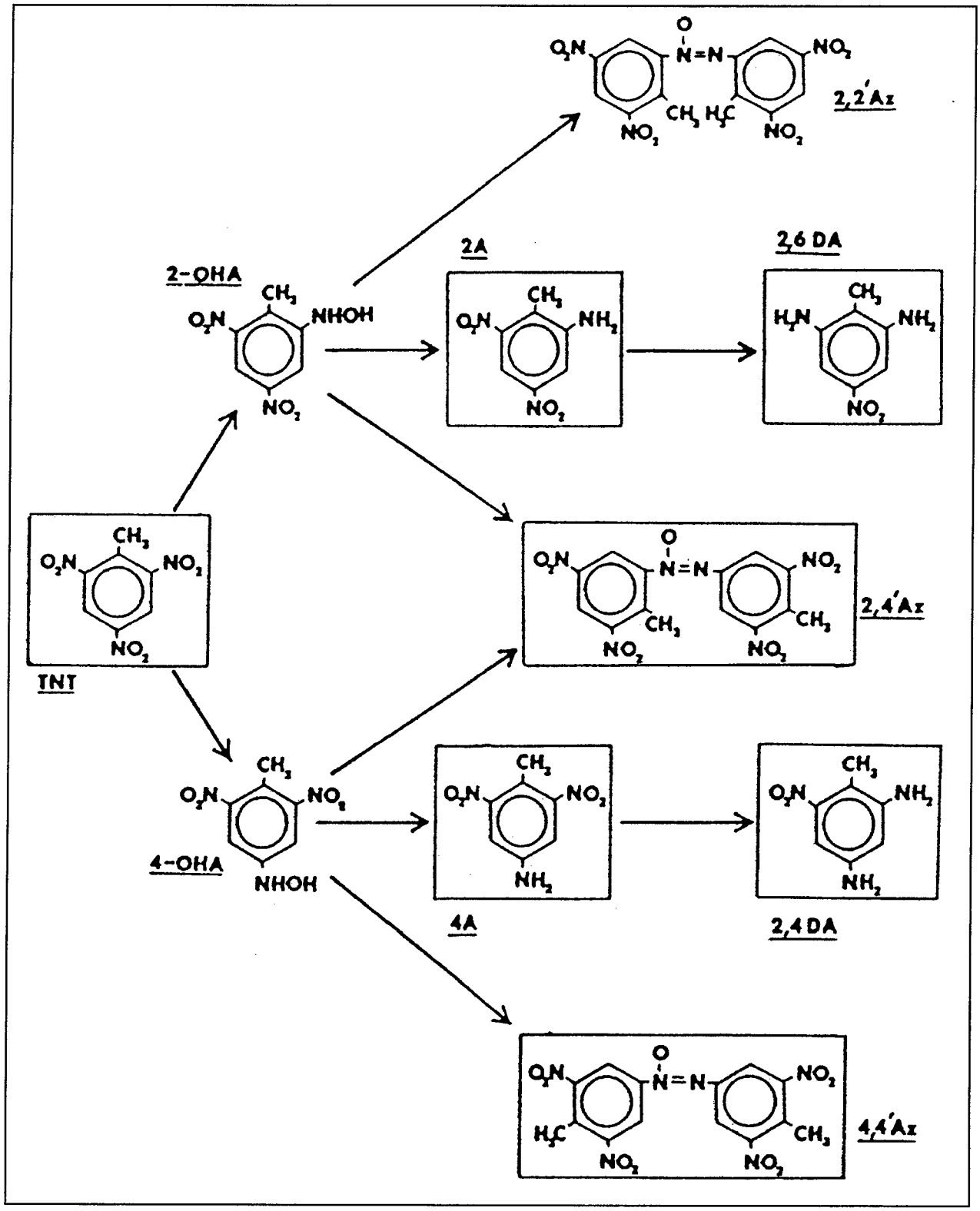

Figure 1. Microbial transformation pathway for TNT (Kaplan and Kaplan 1982)

dimers has also been reported (McCormick, Feeherry, and Levinson 1976; Kaplan and Kaplan 1982; Spanggord, Stewart, and Riccio 1995). The reduction pathways for TNT (Figure 1) appear to be essentially the same in a wide diversity of species and environmental systems including mammals, higher and lower plants, bacteria, soil, activated sludge, and compost; although, the pre dominant amino isomer is 4ADNT in aerobic systems and 2ADNT in anaerobic systems (Carpenter et al.1978; Pennington 1988; Harvey et al.1990).

The objectives of this work were to evaluate various analysis methods to differentiate between free and conjugated explosives and their transformation products in composted soil and digester sludge and to investigate the time course of transformation and conjugation.

Materials and Methods

Composts and digester sludges

Compost samples from aerated windrow composting pilot studies and full-scale treatments at Umatilla Army Depot Activity, Hermiston, OR, were obtained from Black and Veatch Waste Sciences, Tacoma, WA. The compost-amendment mixture was composed of (percent by volume) 30-percent cow manure, 25.4-percent sawdust, 25.4-percent alfalfa, 14.3 -percent chopped potato waste, and 4.9-percent chicken manure. The final compost mixture consisted of 70-percent amendments and 30-percent explosives-contaminated sediment excavated from a dried-out explosives waste lagoon. Samples of composted amendments that contained 10-percent uncontaminated sediments were also available as controls. Digester sludge was from pilot-scale studies performed by Remediation Technologies (Seattle, WA) at U.S. Naval Submarine Base, Bangor, WA. Anaerobic, slurry digestion was performed in lined pits. Contaminated soil, water, an organic carbon source (potato starch), and an inoculum of microbes were mixed in proportions to create a slurry that allowed continuous mixing and heating to above 20 °C. Oxygen initially present in the system was soon consumed, and reducing conditions of less than -100 mV were maintained for 90 days (Tuomi 1995).

Analytical methods

Solvents used for extractions and analysis were HPLC grade from Alltech Associates, Inc. (Deerfield, IL). Concentrated H_2SO_4, Na_2HPO_4 and NaOH were reagent grade from J. T. Baker, Inc. (Phillipsburg, NJ). Solid-phase extraction cartridges (Sep-Pak $Poropak_{RDX}$) were from Waters Corp. (Millford, MA).

HPLC analysis was performed using either a Spectra-Physics system (8875 autosampler, 8800 pump, 8490 detector, Hewlett-Packard 3396 integrator) (San Jose, CA) or a Waters system (717 autosampler, 616 pump, 600S controller, 996 photodiode array detector, Millenium workstation) (Medford, MA). A Phenomenex (Torrence, CA) Ultracarb 5 ODS(20) (4.6 by 250 mm, 5 μm)

reverse-phase column with an Alltech Associates, Inc. (Deerfield, IL), C-18 guard cartridge was used for the analytical separations. The aqueous/methanol (volume %/volume %) gradient elution time steps were as follows: start at 85/15, ramp to 65/35 at 8 min, ramp to 42/58 at 10 min and hold for 13 min, ramp to 0/100 at 28 min and hold for 7 min, ramp down to 85/15 at 40 min and hold for 10 min before the next injection. The flow rate was 0.8 ml min^{-1}. Quantification was by peak height performed at 254 nm, while peak identities and purities were assessed by comparing sample and standard UV spectra (200-400 nm) from a diode array detector, and retention times. Further confirmation of analyte identities was performed using a Supelco (Bellefonte, PA) CN (4.6 by 250 mm, 5 µm) cyanopropyl column that separates the analytes in a different order than the ODS column. The detection limits for the analytes were approximately 0.1 mg/kg of dry compost.

Standards for HPLC analysis were made from Standard Analytical Reference Materials obtained from the U.S. Army Environmental Center (Aberdeen, MD) except for 2,4DANT and 2,6DANT, which were supplied by Dr. Ronald Spanggord, SRI International (Menlo Park, CA).

Solvent extraction

Compost samples were air-dried overnight, then ground to pass a 2-mm screen. Digester sludges contained about 50-percent solids by weight and had become aerobic by the time of analysis. Sludge samples were air-dried overnight prior to analysis. Subsamples weighing 2.00 g were added to 40-ml glass vials that were sealed with Teflon-lined caps. A 10-ml aliquot of HPLC-grade acetonitrile was added and the sample mixed on a vortex mixer for 1 min. The vials were placed into an ultrasonic bath and sonicated overnight at 30 °C. After sonication, a 10-ml aliquot of aqueous calcium chloride solution (5 g L^{-1}) was added to hasten flocculation, the sample was vortexed briefly, and centrifuged at 2000 g for 5 min. The aqueous $CaCl_2$/acetonitrile extract was decanted, filtered, and saved for HPLC analysis. The vials were refilled with fresh $CaCl_2$/acetonitrile solution, vortexed, centrifuged, and decanted repeatedly (usually three times was adequate) until no more unbound explosives residues or free amino transformation products were detectable by HPLC. The resulting solvent-extracted residues were decanted and air-dried overnight before hydrolysis experiments were initiated.

Acid hydrolysis

The first of two hydrolysis procedures was an acid hydrolysis. An air-dried residue from the acetonitrile extraction was transferred to a 22-ml vial, 10 ml of 50-percent aqueous H_2SO_4 was added, the sample was vortexed for 1 min, then placed into an ultrasonic bath for 6 hr at 30 °C. Following sonication, the vial was centrifuged at 2,000 g for 5 min and a 5-ml aliquot of the acid digest removed and neutralized by adding 100 ml of aqueous, 1.2 M Na_2HPO_4 (pH 8.4). The resulting solution (pH 6.5) was pulled through a 6-ml Sep-Pak Porapak$_{RDX}$ cartridge at 10 ml min^{-1} using vacuum. The cartridge was washed with an additional 20 ml of reagent-grade water to remove salts, then evacuated for 5 min to remove residual water. A 5-ml aliquot of acetonitrile was added and allowed to

drip through the cartridge at 5 ml min^{-1}. Reagent-grade water (5 ml) was washed through the cartridge into the acetonitrile extract. The mixture was diluted to 10.0 ml using reagent-grade water and analyzed by HPLC.

Base/acid hydrolysis

The second hydrolysis procedure combined a base and acid hydrolysis. Solvent-extracted and air-dried samples from the Day-15 compost and a time series of the digester sludges were transferred to 22-ml vials and treated with 10 ml of 0.5 M NaOH. The samples were vortex mixed for 3 min and sonicated overnight at 30 °C. The vials were centrifuged, and a 5-ml aliquot of each basic extract of the Day-15 compost was removed and diluted to 10 ml with concentrated H_2SO_4. The remaining 5 ml of basic extract was left in the vial with the residue and 5 ml of concentrated H_2SO_4 was added. Acidified residues were sonicated for 6 hr at 30 °C, centrifuged, and 5-ml aliquots removed and neutralized with 1.2 M Na_2HPO_4. As a precaution, 3,5-dinitroaniline (3,5DNA) was added as a surrogate to the neutralized digests to monitor recovery from the Sep-Pak cartridges.

Acid hydrolysis of base-hydrolyzed residues was modified to eliminate the spattering of digestion mixture when concentrated acid was added too quickly. A sample of Day 10 compost was solvent-extracted, rinsed, and base/hydrolyzed as above. A 5-ml aliquot of -15 °C, 50-percent H_2SO_4 was added to the 5 ml of base hydrolysis mixture. The heat generated using this procedure did not cause spattering.

Treatment of ^{15}N-labeled conjugates

Freeze-dried samples (400-500 mg) of whole peats and humic acids that had been incubated with ^{15}N-labeled ADNT and DANT isomers were received from Dr. Kevin Thorn (USGS, Arvada, CO). Subsamples (3-6 mg) were solvent extracted with 500 µL of acetonitrile. The residues were then base/acid hydrolyzed using 0.5 M NaOH and 50-percent H_2SO_4. The residues were rinsed with reagent-grade water, air-dried, and returned to Dr. Thorn for further ^{15}N NMR analysis.

Prior to ^{15}N NMR analysis, unreacted ADNTs and DANTs were removed from the whole peats by dialysis and from the humic acids by cation-exchange, solid-phase extraction. Samples of the dialysates and solvent-eluted cation-exchange columns were analyzed to determine the quantities of unreacted starting materials. NMR results are described in Chapter 2.

Recoveries

Spike/recovery experiments were performed by adding seven explosives and transformation products dissolved in acetonitrile to field-moist composts, digester sludge, and sand. The spiked concentrations ranged from 1 to 3 mg kg^{-1} (dry

material) for one set of triplicate samples and 25 to 75 mg kg^{-1} for an additional unreplicated set. The acetonitrile was evaporated in a few minutes as the mixtures were homogenized. The air-dried samples were extracted with acetonitrile in accordance with the procedure described earlier.

Neutralization of acid digests of residues sometimes produced a white, gelatinous precipitate. A spike-recovery experiment was performed to determine the relationship between final pH and recovery. Residues from solvent-extracted compost samples were spiked at 16 mg kg^{-1} with the ADNTs and the DANTs. The samples were immediately base/acid hydrolyzed. The hydrolysates were subdivided and neutralized to either pH 4.0, 5.0, or 6.0. The 3,5DNA surrogate was added, and the neutralized hydrolysate passed through the solid-phase extraction cartridges.

To evaluate the recovery of the base/acid-hydrolysis procedure, 0.5 M NaOH was added to two solvent-extracted compost samples and sand. Either the initial explosives (HMX, RDX, TNT) or the TNT transformation products were spiked into the mixtures at concentrations ranging from 25 to 75 mg kg^{-1}. The mixtures were sonicated overnight, then brought to 25-percent acid by adding -15 °C, 50-percent H_2SO_4 followed by sonicating again for 6 hr. Hydrolysates were neutralized to pH 5.0 with Na_2HPO_4. The 3,5DNA surrogate was added immediately before solid-phase extraction. An aqueous base solution with no added solids was also treated with the same procedure.

Results and Discussion

Solvent extraction

The exhaustive, acetonitrile extractions of the time series showed that the concentration of solvent-extractable (free) TNT was rapidly reduced in compost treatments from 2,370 mg kg^{-1} on Day 1, to 28.6 mg kg^{-1} on Day 10, and to <5 mg kg^{-1} by Day 20. These results agree with previous analyses of the composts (Caton et al. 1994; Pennington et al. 1995). The ADNTs decreased with time, while the DANTs increased for 10 days before decreasing (Table 1, Figure 2). Trace levels of the azoxy transformation products of TNT and the nitroso transformation products of RDX were found in a few of the samples. No hydroxylaminodinitrotoluenes were detected.

When $CaCl_2$/acetonitrile was decanted from samples being extracted, about 10 percent of the solvent was retained by the residues. Each repeat decantation with fresh solvent removed about 90 percent of the previously retained analytes, but no further dissolution from the solids was evident. Thus, repeat decantations

Table 1
Concentrations (mg kg^{-1}) in Compost from Umatilla Windrows Over Time[1] (percent relative standard error)

		Aminodinitrotoluenes		Diaminonitrotoluenes				Nitramines		Mono-nitroso-	Trini-troso-
	TNT	4ADNT	2ADNT	2,6DANT	2,4DANT	TNB	2,4DNT	HMX	RDX	RDX	RDX
Day 1 ACN[2]	2,370 (<1)	291 (8)	170 (8)	<0.1	<0.1	8.4 (7)	3.3 (4)	219 (14)	1,030 (10)	<0.1	<0.1
Day 1 ACID[3]	<0.1	23.6 (9)	4.6 (9)	<0.1	15.2 (25)	<0.1	<0.1	<0.1	4.4 (25)	<0.1	<0.1
Day 5 ACN	1,050 (10)	210 (73)	220 (12)	<0.1	<0.1	21.2 (10)	2 (16)	211 (8)	982 (6)	<0.1	<0.1
Day 5 ACID	<0.1	31.2 (140)	4.7 (8)	1.6 (4)	34.4 (10)	<0.1	<0.1	<0.1	3.1 (18)	<0.1	<0.1
Day 10 ACN	28.6 (5)	149 (6)	20.3 (5)	5.3 (16)	53.4 (23)	<0.1	<0.1	217 (5)	546 (4)	<0.1	<0.1
Day 10 ACID	<0.1	63.1 (4)	5.1 (8)	4.5 (2)	75.1 (9)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Day 15 ACN	14.7 (2)	24.5 (3)	2.2 (4)	<0.1	8.3 (15)	<0.1	<0.1	123 (2)	73.9 (2)	3.9 (7)	2.1 (19)
Day 15 ACID	<0.1	46.4 (2)	3.7 (1)	4.2 (19)	66.1 (5)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Day 20 ACN	1.6 (25)	9.2 (5)	1.3 (4)	<0.1	4.6 (37)	<0.1	<0.1	80.1 (3)	19.3 (15)	2.8 (9)	3.5 (2)
Day 20 ACID	<0.1	21.2 (3)	2 (1)	2.4 (1)	53.1 (1)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Day 40 ACN	3.3 (14)	13.3 (12)	23 (10)	<0.1	<0.1	<0.1	<0.1	67.8 (13)	16.4 (11)	5.8 (11)	4.5 (1)
Day 40 ACID	<0.1	31.4 (7)	3.4 (1)	2.6 (20)	34.4 (4)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[1] Values represent means of three replicates.
[2] Acetonitrile extraction by overnight sonication.
[3] Hydrochloric acid extraction by 6-hr sonication.

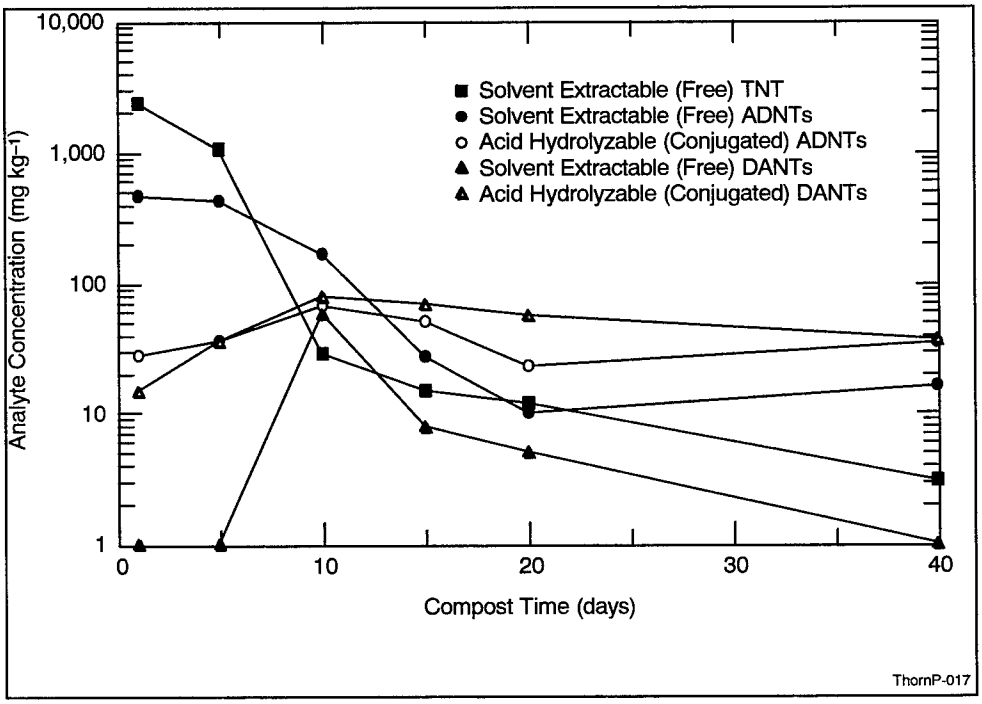

Figure 2. Concentration of free (solvent-extractable) TNT and free and conjugated (acid-hydrolyzable) transformation products recovered from aerated compost

served only to purify the residue, removing TNT, RDX, and "free" ADNTs and DANTs. This finding agrees with earlier work from Myers. [1]

The precision of acetonitrile extraction, based on percent relative standard deviation (percent rsd) estimates for triplicate determinations showed no clear dependence on either analyte or concentration. Values ranged from <1 to 73 percent. The high value was an isolated case since the two next highest values were 37 and 25 percent. Excluding the one high value and the several cases where <1 percent was found, the remaining 42 estimates gave a pooled rsd of 11.9 percent with 84 deg of freedom.

Acid hydrolysis

On the first day after initiation of composting, conjugated ADNTs and DANTs had already been released by the acid hydrolysis (Figure 2). The quantities of conjugated transformation products increased for 10 days, then gradually decreased to levels that were approximately equal to, or slightly higher than, the Day 1 level. Samples of this compost, which had been stored in large plastic bags at ambient temperature for several months, were also analyzed. After this aging, the quantities of aminodinitrotoluenes and diaminonitrotoluenes that were released by hydrolysis were further reduced from the Day 40 compost (from 37.8 to 8.1 mg kg^{-1} and from 38.0 to 5.51 mg kg^{-1}, respectively), demonstrating that further conjugation of these bound residues continues to occur over time.

Acid hydrolysis of solvent-extracted residues degrades the matrix substantially, but results indicated that no additional TNT, RDX, or HMX was released. The stability of TNT, RDX, HMX, and the ADNTs under conditions used for acid hydrolysis has been demonstrated using compost residues. These results confirmed that solvent extraction removes all free TNT, RDX, and HMX from sorption sites deep within the matrix as previously shown for soils (Jenkins and Grant 1987) and confirms the results for compost obtained by Pennington et al. (1995).

Base/acid hydrolysis

Base/acid hydrolysis experiments were performed using Day 15 aerated compost and a time series of digester sludge. The quantity of DANTs released from the Day 15 compost by the base hydrolysis was roughly equal to the amount released by the acid-only hydrolysis (57.2 mg kg^{-1} compared with 70.3 mg kg^{-1}). The base hydrolysis released less of the ADNTs than were released by the acid hydrolysis (19.8 mg kg^{-1} compared with 50.1 mg kg^{-1}). However, the subsequent acid hydrolysis of the base-hydrolyzed residues released considerably more of the conjugated transformation products (88.8 mg kg^{-1} of ADNTs and 284 mg kg^{-1} of DANTs) (Figure 3). The combination of base/acid hydrolysis increased the total recovery of conjugated ADNTs and DANTs by about a factor of three over acid hydrolysis alone.

[1] Personal Communication, 1998, K. Myers, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

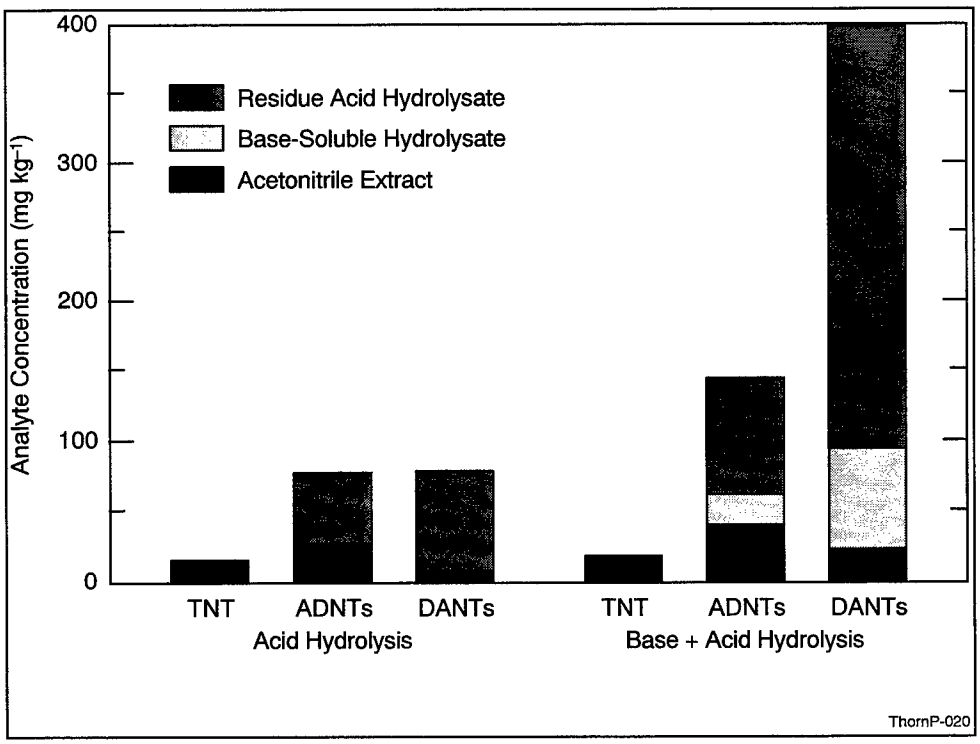

Figure 3. Comparison between recoveries of ADNT and DANT transformation products from Day 15 aerated compost using acetonitrile extraction, acid digestion, and base/acid digestion

Results from acetonitrile extraction of the digester time-series sludges (Figure 4) agree with previous determinations (Tuomi 1995). Analysis of the extracts and hydrolysates revealed a pattern of explosives degradation and transformation product conjugation that was similar to that found in the composts.

Control composts (composts with the same starting materials, but free of explosives residues) were also extracted, hydrolyzed, and analyzed. Retention times and spectra of all peaks, including the unretained solvent peak, were compared with those from sample composts. No unidentified TNT transformation products were released by hydrolysis.

^{15}N-labeled conjugates

Prior to ^{15}N NMR analysis, the whole peats had been dialyzed to remove unreacted starting material. One analysis of three sequential dialysates of the 2,4DANT indicated that this procedure was sufficient. Solvent extraction of the whole peats and humic acids confirmed that only small quantities of unreacted 4ADNT and 2ADNT (8 and 13 mg kg^{-1}, respectively) or 2,4DANT (0.6 13 mg kg^{-1}) remained. Base/acid hydrolysis of the samples did not release additional quantities of conjugated transformation products.

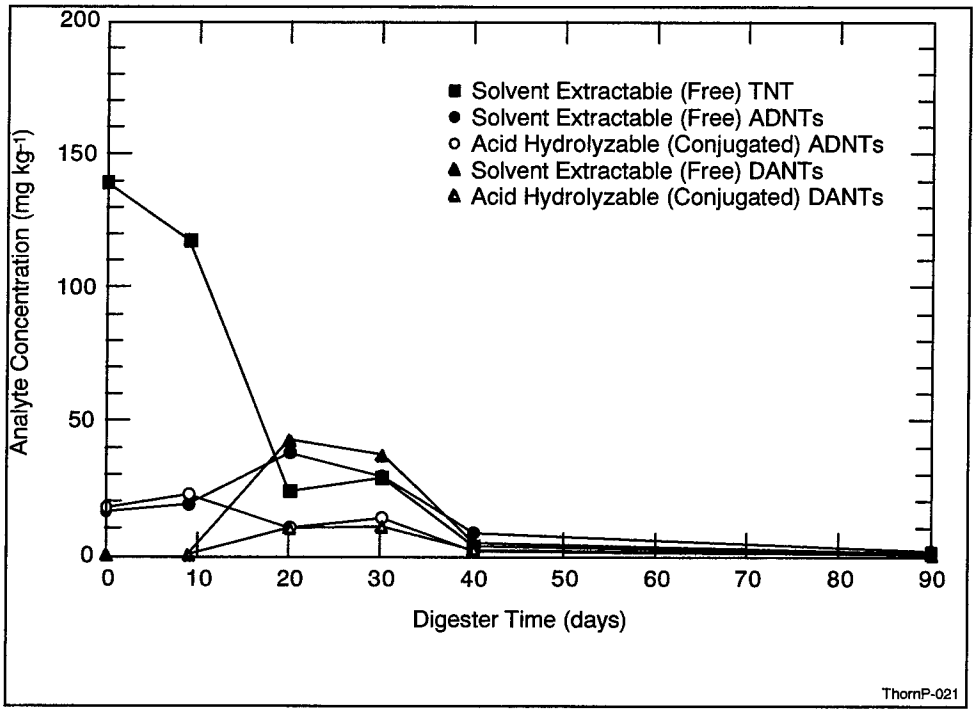

Figure 4. Concentration of free (solvent-extractable) TNT and free and conjugated (base/acid-hydrolyzable) transformation products recovered from digester sludge

Recoveries

The acetonitrile extractions of sand produced recoveries of RDX, HMX, TNT, and 2ADNT from 92 to 97 percent (Table 2). Recovery of 4ADNT was 82 percent. The DANTs were recovered at less than 27 percent. The recoveries from compost were greater than 94 percent for the nitramines (RDX and HMX); however, the recovery of TNT dropped to 35 percent. This suggests that TNT is transformed and conjugated rapidly by compost. The recoveries of the transformation products were similar to the recoveries from sand, but these values could be inflated by material initially added as TNT. Recoveries from digester sludge were 90 percent and above for the nitramines and 88 percent for TNT. The recoveries of the TNT transformation products were less than 40 percent for the aminodinitrotoluenes and less than 21 percent for the diaminonitrotoluenes. Reproducibility for these recovery estimates was best in sand (rsd = 5.0 percent) and about 10 percent for compost and digester sludge. When the compost was spiked at concentrations ranging from 25 to 75, the 13-mg kg^{-1} recoveries of the TNT and diaminonitrotoluenes rose to greater than 69 percent, while the recoveries of the aminodinitrotoluenes were less than 82 percent. The difficulty in recovering ADNTs, even in acidified water matrices, has been reported previously (Jenkins, Thorne, and Myers 1995).

Table 2
Percent Recoveries of Spikes by Acetonitrile Extraction (percent relative standard deviation)[1]

		Aminodinitrotoluenes		Diaminonitrotoluenes		Nitramines	
	TNT	4ADNT	2ADNT	2,6DANT	2,4DANT	HMX	RDX
Compost (low)[2]	35 (16)	91 (8)	88 (11)	45 (9)	27 (12)	94 (7)	97 (11)
Digester sludge	88 (10)	25 (8)	40 (40)	21 (13)	7 (12)	103 (7)	90 (2)
Sand	93 (3)	82 (1)	97 (3)	27 (8)	11 (9)	92 (2)	97 (2)
Compost (high)[3]	72	58	82	92	69	82	103

[1] Values represent means of three replicates except where noted.
[2] Compost spiked at 1-3 mg of various analytes kg^{-1}.
[3] Compost spiked at 25-75 mg of various analytes kg^{-1} without replication.

Careful control of pH was necessary (Table 3). A pH of 5.0 is recommended to provide a compromise between absolute recovery of all analytes and a reasonable recovery of the 3,5DNA surrogate so that solid-phase extraction recoveries can be estimated independently of the hydrolysis/neutralization losses.

Table 3
Effect of Neutralization pH on Percent Recovery of Hydrolized Spikes

	Aminodinitrotoluenes		Diaminonitrotoluenes		Surrogate
	4ADNT	2ADNT	2,6DANT	2,4DANT	3,5DNA
Compost A					
pH4	<0.4	<0.4	14	42	74
pH5	<0.4	<0.4	22	76	58
pH6	<0.4	<0.4	19	76	10
Compost B					
pH4	<0.4	<0.4	10	29	75
pH5	<0.4	<0.4	12	39	51
pH6	<0.4	<0.4	8	29	28

Acid hydrolysis from addition of cold 50-percent acid resulted in lower recoveries of TNT transformation products than were recovered when 100-percent sulfuric acid was used (Table 4). Nevertheless, the use of 50-percent acid is recommended because of the potential for injury due to spattering of hot acid when 100-percent acid is used. These analytical recoveries should be as sessed in the context of the dynamic nature of compost and sludge systems. The DNT and DANT transformation products of TNT are formed, conjugate, and further bind as biotreatment takes place. Target analytes spiked into these matrices are unstable, and recoveries are a function of the evolution of these processes.

Table 4
Recovery of Transformation Products from Compost Using Cold 50-Percent Sulfuric Acid After Base Hydrolysis

	Concentration, mg/kg			
	Aminodinitrotoluenes		Diaminonitrotoluenes	
	4ADNT	2ADNT	2,6DANT	2,4DANT
100% acid	63.1	5.1	4.5	75.1
50% acid	19.8	<0.1	3.08	97.1

None of the untransformed explosives TNT, RDX, and HMX were detected in the hydrolysis spike recovery tests due to their instability in basic solution. Results showed that the procedure recovered from 73 to 90 percent of the ADNTs and from 88 to 93 percent of the DANTs when no solids were present (Table 5). However, in the presence of compost, no ADNTs were detected; the recoveries of the DANTs dropped to 18-62 percent. In the presence of sand, the recovery of the ADNTs ranged from 5 to 9 percent while the recovery of the DANTs was from 9 to 40 percent. For all mixtures that contained solids, the recovery of the 3,5DNA surrogate that was added to the neutralized hydrolysates immediately before solid-phase extraction ranged from 72 to 88 percent.

Table 5
Percent Recoveries of Explosives Transformation Products (percent relative standard deviation)[1]

	Aminodinitrotoluenes		Diaminonitrotoluenes		
Matrix	**4ADNT**	**2ADNT**	**2,6DANT**	**2,4DANT**	**Surrogate**
Solution phase from base/acid hydrolysis	90 (3)	73 (11)	88 (11)	93 (11)	106 (8)
Compost A	<0.1	<0.1	18 (21)	62 (22)	72 (14)
Compost B	<0.1	<0.1	39 (32)	56 (5)	87 (4)
Sand	9 (1)	5 (1)	9 (1)	40 (6)	88 (5)

[1] Values represent means of three replicates spiked with 25-75 mg of each of sevenanalytes kg^{-1}. Analytes included TNT, RDX, HMX, 4ADNT, 2ADNT, 2,6DANT, 2,4DANT, and the surrogate, 3,5DNA.

The ability to hydrolyze and recover unbound transformation products from composts changes over the time course of the treatment. Hydrolysis of the aerated composts released the greatest quantities of TNT transformation products during the midpoint of the composting process (Table 1). Hydrolysis of the Day 40 compost released less of the transformation products, presumably due to further conjugation with formation of functional groups that are more resistant to hydrolysis. The incomplete recoveries of TNT and transformation products that were spiked to composts from the end of full-scale operations indicated that fully developed compost has a residual capacity to reduce TNT and conjugate the resulting transformation products. This does not explain the loss of transforma tion products in the presence of sand with negligible organic carbon (<0.2 percent), however, but is consistent with losses of these compounds reported for acidified water

(Jenkins, Thorne, and Myers 1995). None of the azoxy transformation products of TNT were detected.

Conclusions

The results of this study were consistent with earlier findings of immobili za-tion of TNT transformation products (Caton et al. 1994; Pennington et al. 1994; Comfort et al. 1995). HPLC analysis of hydrolysates has identified some hydrolyzable products.

The transformation of TNT to solvent nonextractable transformation products goes through two stages of covalent conjugation in compost and digester sludge. In the first stage, about 20 percent of the transformed TNT can be released through hydrolysis. As processing continues, a second stage occurs in which the bonds are either altered to form different functional groups that are nonhydrolyzable, or additional bonds form as the bound transformation products are further reduced and conjugated through multiple bonds.

Spike-recovery studies indicated that current methods do not adequately quantify TNT transformation products in soils, particularly at the low concentra tions that characterize remediation goals. Current methods are sufficient to remove all of the analytes that are extractable. These may consist of the explosives remaining undissolved in the soil, plus the pool of transformation products that are in the aqueous phase or sorbed and not yet conjugated to the solid phase of the organic matter. However, the transformation products that are released by hydrolysis represent a dynamic pool of weakly conjugated amines that are subject to further reduction and/or bond transformations that eventually become unhydrolyzable as humification continues. Therefore, analyses of bioremediation matrices should be considered as a qualitative descriptor of the progress of humification and the capacity to covalently conjugate transformation products than as a quantitative measure of the absolute amounts of various analytes present.

The purpose of the base/acid hydrolysis is to break the bonds between TNT transformation products and organic carbon biopolymers such as humic acid. A minimum of 0.1 mg of transformation product would have to be released from 1 kg of organic carbon to be detected. Therefore, the method is only applicable to high-carbon matrices like compost, digester sludge, or plant and animal tissues. In soil or aquifer materials that contain a few percent or less of organic carbon, the quantity hydrolyzable products potentially available for analysis are insufficient for determination by this method.

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4 Microbial Degradation of Conjugated Fractions

Introduction

Background

The long-term stability of the residues of TNT in composted soils and the potential for release of environmentally undesirable products depend upon the microbial degradability of finished compost. Current evidence supports immobilization of TNT transformation products to more complex residuals, rather than mineralization to simple nonhazardous compounds such as carbon dioxide and nitrate. The bioavailability of these complex residuals to further microbial degradation is unknown. Therefore, the microbial degradation potential for finished compost and for separate fractions of finished compost were evaluated by challenging these residuals with microorganisms isolated from the compost. The isolates, obtained from enrichment cultures, were reintroduced to the compost to increase the potential for degradation. Radiolabeled TNT was introduced into the soil prior to composting to serve as a marker for mineralization and degradation activity.

Objectives

Objectives were to evaluate the ability of soil microorganisms to degrade radiolabeled TNT-derived residues bound to various organic components in the compost and determine the mechanisms limiting or supporting the degradation process.

Materials and Methods

Microbial cultures enriched on cellulose were examined for their ability to release $^{14}CO_2$ from compost prepared with [^{14}C]TNT-amended soil. Two additional studies were conducted to evaluate factors responsible for the ability of microbial isolates to attack TNT-derived residues in whole compost and compost fractions. The ability of isolates from cellulose and whole compost to mineralize uncomposted TNT was evaluated. To evaluate the role of nutrient limitations on degradation,

nitrogen and phosphorus were added to some treatments containing each of the microbial isolates.

Isolation of microorganisms from whole compost

Procedures used for isolation of microorganisms able to grow on whole compost were the same as those used for conjugate fractions (Pennington et al. 1997). However, in the case of whole compost, 0.5 g of whole compost with unlabeled TNT as used together with 9.5 ml of mineral salts medium. Following three monthly transfers, isolates were obtained by streaking onto a mineral salts medium (MSM) containing whole compost (the formulation for MSM was described previously (Pennington et al. 1997)). Isolates were analyzed by comparison of cell lipids with those of known bacteria and by the MIDI Labs bacterial identification system using 16S rRNA gene sequencing (MIDI Labs, Newark, DE).

Assay of radiolabeled whole compost and compost fractions

Combinations of isolates having the greatest capacity to mineralize or transform the conjugation products were challenged with the radiolabeled whole compost. Mineralization activity was compared with previous findings by conducting studies using the same procedures. The microbial isolates were from the cellulose and fulvic acid fractions, each containing ^{14}C-TNT-derived residues. Groups of isolates were as follows: (a) five isolates obtained from individual compost fractions (cellulose, humin, humic acid, fulvic acid, acetonitrile extract) (Pennington et al. 1997); (b) a single isolate from whole compost; or (c) all six isolates (i.e., groups a and b above). Microorganisms were also incubated for 30 days with whole compost in a mineral salts solution. Evolved $^{14}CO_2$ trapped in 1N KOH was assayed at 3, 7, 14, 21, and 30 days (Pennington et al. 1997). Samples of the radiolabeled whole compost slurry were taken at the end of incubation and extracted for lipids. The lipid analysis was conducted to determine what changes, if any, had occurred in the composition of the groups and to determine if any of the ^{14}C was taken up and metabolized by the microorganisms. Both autoclaved (sterile) and unautoclaved (nonsterile, containing microorganisms associated with the processed compost, but without added inoculum) flasks were used as controls.

To determine whether or not cellulose and whole compost isolates could degrade unconjugated TNT while growing on whole compost, fresh ^{14}C-TNT (0.2 μCi) was added to finished compost that had been prepared with 39.1 mg of unlabeled TNT kg^{-1} soil. Slurries inoculated with the cellulose or whole compost isolates were also amended with either 0.1 mM NH_4Cl or 0.05 mM K_2HPO_4 in the presence of the native compost population.

Effects of environmental factors

The impact of pH on growth of each of the isolates (whole compost and compost fraction) was assessed using MSM containing 8 g of nutrient broth L^{-1} and adjusted to pH 5, 6, or 7. Cultures were incubated for 3 days at 23 °C and scored

for changes in turbidity relative to an uninoculated control. The effect of temperature on growth of each of the isolates was determined using MSM containing 8 g of nutrient broth/L at pH 6.0. and incubating the inoculated cultures at 22, 27, and 32 °C for 3 days. As for the pH evaluation, the impact of temperature on growth was estimated by measuring changes in turbidity.

Lipid extracts

Extraction. One to two grams of homogenized compost was weighed into a 15-ml test tube (minimum of three replicates per analysis). An aliquot of extractant, methylene chloride:methanol:phosphate buffer (monobasic, pH 7.0) (1:2:0.8, v:v:v), was added, 3.8 ml for 1 g of compost and 7.6 ml for 2 g. The compost extractant mixture was subjected to a 2-min period of sonication followed by a 2-hr period of extraction. The mixture was centrifuged on a table top unit, decanted into a clean test tube, and the compost washed with another 1 to 2 ml of methylene chloride. To the combined whole compost extract and wash, 1 to 2 ml (for 1 g or 2 g of compost, respectively) of water was added and the sample mixed thoroughly, followed by centrifugation as before. A two-phase system was formed. The upper or aqueous methanol phase contained polar nonextractable materials. The lower or solvent extract phase contained the lipids, glycolipids, and nonpolar lipids. The lower phase containing the lipids was removed by pipetting, placed into a clean test tube, and taken to dryness under a gentle stream of nitrogen.

Fractionation. The dried extract was redissolved in 100 µl of methylene chloride and applied to the top of a prewashed silica gel column (Burdick and Jackson, Muskegon, MI). To ensure complete transfer of the extract to the column, an additional two 100-µl washes of methylene chloride were applied. The extract was fractionated into three parts: nonpolar, where most contaminants are found; moderately polar, where many plant and some bacterial cellular components are found; and polar, where bacterial membrane lipids (polar lipid fatty acids or PLFA) are found.

Derivatization. The methanol eluant was evaporated to dryness under N_2, dissolved in 1.0 ml of DCM:MeOH (1:1, v:v) and 1 ml of 2.0 M methanolic KOH (1:1, v:v), and heated at 40 °C for 30 min to form the fatty acid methyl esters of the recovered phospholipids (PLFAME). Upon cooling, the PLFAME were extracted in 2 ml of hexane:DCM (4:1, v:v) after additions of 0.2 ml 1N glacial acetic acid and 2 ml of water.

Explosives chemical analyses

For explosives analysis, 1 to 2 g of homogenized compost was quantitatively extracted in a 15-ml test tube with the methylene chloride:methanol:phosphate buffer extractant as for the lipid extraction described above. However, the only difference was that the compost-extractant mixture was sonicated for 18 hr prior to separation by centrifugation. The combined extract and wash (1 to 2 ml of water for 1 to 2 g of compost, respectively) were mixed thoroughly and then centrifuged. The

lower extractant phase was removed by pipetting and taken to dryness under nitrogen, redissolved in 100 μL of methylene chloride, and passed through a prewashed silica gel column (Burdick and Jackson, Muskegon, MI) together with two additional 100-μL washes. The explosives moved through the column with the methylene chloride (nonpolar or neutral lipid phase), and the methylene chloride-explosives phase was again taken to dryness with nitrogen. The extract was then dissolved in 150 μL of acetonitrile and diluted with one part distilled water to give a 50:50 solvent:water mixture. A 25-μL aliquot of this mixture was placed onto either a Hewlett Packard 9010 HPLC with a UV detector run at 234 and 254 nm (Hewlett Packard Instruments, Inc., San Fernando, CA) or a Waters Millenium HPLC using a photodiode detector (Waters Instruments, Inc., Milford, MA). Separations on both instruments were performed on an ODS Hypersil Reverse Phase 100-by 4.6-mm column (Hewlett Packard Instruments, Inc., San Fernando, CA) using a mobile phase of 68 percent 20 mM ammonium chloride to 32 to 98 percent methanol:2 percent butanol under isocratic conditions for the 15-min run.

Data analysis

PLFA and/or contaminants were identified by retention time comparisons with authentic standards (Supelco, Bellefonte, PA). Alternatively, a mass selective detector interfaced to the gas chromatograph was used to provide accurate identification on a compound-to-compound basis. Prior to injection, an appropriate amount of internal standard was added to each test tube. The sample was dissolved in hexane that contained an internal standard (nonadecanoic acid) at a concentration of 15.6 ng μL^{-1}. Sufficient hexane to produce a chromatograph in which 10 percent of the peaks were full scale was considered appropriate for each analysis. Typically, 1 g of compost extract dissolved in 100 μL of internal standard resulted in a satisfactory chromatogram. Internal standard calculations were then used to obtain compound concentrations.

Results and Discussion

Nature and characteristics of microbial isolates

Only one microorganism was isolated from each compost fraction (Pennington et al. 1997). Although each fraction was inoculated with a medium rich in microbial diversity (garden soil), 2 months of incubation resulted in the survival of only one culturable microorganism. These results suggest that whole compost and the individual compost fractions do not support microbial growth in general.

A total of six isolates were recovered from whole compost and inoculated compost fractions (Table 1). Three isolates were identified as gram-negative, two as gram-positive, and one as gram-variable bacteria, based on Gram stain and growth on MacConkey's medium, which is selective for gram-negative species.

Table 1
Bacterial Isolates Recovered from Whole and Fractionated Compost Material

Source	Abbreviation	Gram Stain	McConkey's[1]
Whole compost	WC	Positive	Negative
Humin	Humin	Positive	Negative
Cellulose	CELL	Negative	Positive
Humic acid	HA	Negative	Positive
Fulvic acid	FA	Variable	Negative
Extractable	ACN	Negative	Positive

[1]Selective for gram-negative species.

Similarities among the six compost and compost fraction isolates and relationships to other standard bacteria were determined by hierarchal cluster analysis of the lipid profiles (Figure 1). The two gram-positive isolates demonstrated relationships to high guanosine + cytosine gram-positive bacterial standards; these were humin isolate with *Arthrobacter globiformis* and *Micrococcus luteus* and the whole compost isolate with *Bacillus subtilis*. While the whole compost and fulvic acid isolates stained gram-positive, each showed a linkage to the gram-negative isolates based on their lipid composition. Thus, both of these bacteria are possibly gram-variable, exhibiting characteristics of both gram-positive and gram-negative bacteria, depending upon the nature of the environment encountered. The gram-variable fulvic acid isolate was unique in its lipid composition. Although the fulvic acid isolate demonstrated a relationship to the *Acinetobacter caligenes* standard, the isolate contained a unique fatty acid, tuberculostearic acid, that typically occurs in the actinomycetes and mycobacteria.

The cellulose, humic acid, and acetonitrile gram-negative isolates grew on MacConkey's medium (Table 1). They had similar lipid profiles and may represent the same genera of bacteria (Figure 1). These three isolates were not closely related to any of the bacterial standards. The humic acid isolate exhibited a single lipid profile closely related to a gram-positive bacterium.

The isolates from the cellulose fraction and whole compost were sent to MIDI Laboratories (Newark, DE) for 16S rRNA gene sequencing to provide a second approach for microbial identification. The resulting data indicated that the cellulose isolate was *Pseudomonas fluorescenes* strain G. However, because the percent difference from the reference stain was not perfect (0.86-percent difference), the highest level of confidence (>95 percent) can only be given to identification at the genus level. By contrast, the whole compost strain was identified as the microorganism *Bacillus thuringensis* strain kurstaki. This identification was a complete match at the species level.

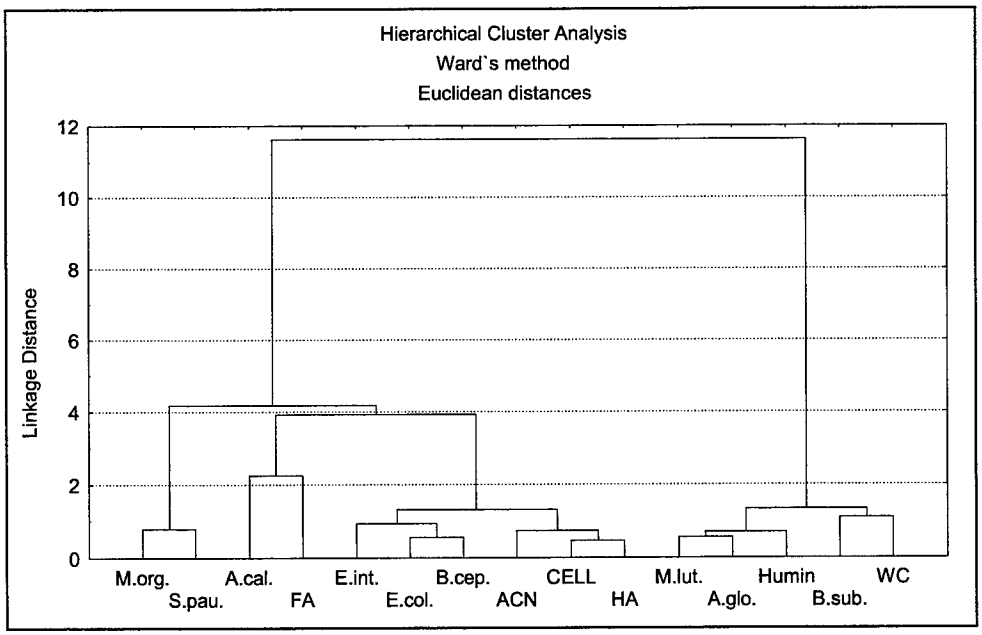

Figure 1. Taxonomic relationships of representative compost isolates (Abbreviations: M. org. = *Morganella organii*, S. pau = *Spingomonas paucimobilis*, A. cal. = *Alcaligenes calcoaceticus*, FA = Fulvic acid isolate, E. int. = *Enterobacter intericum*, E. coli = *Escherichia coli*, P. cep = *Pseudomons cepacia*, ACN = Acetonitrile isolate, CELL = Cellulose isolate, HA = Humic acid isolate, M. lut. = *Micrococcus luteus*, A. glo. = *Arthrobacter glomerus*, Humin = Humin isolate, B. sub. = *Bacillus subtilus*, WC = Whole compost isolate)

Consolidating the above information indicated that six isolates were actually recovered. These were *Bacillus thuringensis* on whole compost (also referred to as isolate WC); an unidentified isolate on humin; a *Pseudomonas* strain, tentatively identified as *fluorescens* strain G on cellulose (also referred to as isolate CELL); and three unidentified isolates on humic acid, fulvic acid, and acetonitrile (also referred to as isolates HA, FA, and ACN), respectively. These are the names of the microorganisms used from this point on in the report.

Mineralization

The $^{14}CO_2$ evolution from cellulose and fulvic acid fractions were consistent with previous results (Pennington et al. 1997). The $^{14}CO_2$ released from the cellulose fraction showed pronounced degradation that was nearly linear and reached a total $^{14}CO_2$ accumulation of 23 percent in 30 days (Figure 2). The nonsterile control (native microflora associated with the cellulose fraction without enrichment) also showed a nearly linear, albeit slow, increase in accumulation over the same time period. The degradation pattern for the fulvic acid cultures generally demonstrated significant differences between the active and nonsterile control cultures (Figure 3).

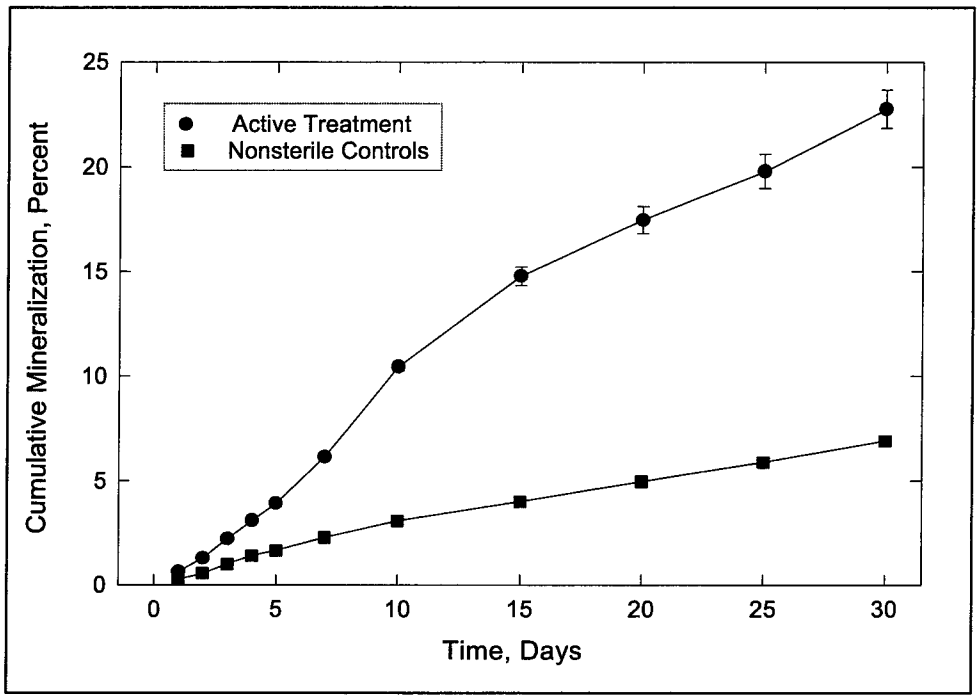

Figure 2. ^{14}C-labeled TNT residue mineralization from cellulose fraction

In contrast to the cellulose results, evolution of $^{14}CO_2$ in the fulvic acid active treatment gradually leveled off after 15-20 days of incubation, never rising substantially above a total accumulation level of 17.6 percent. Since the active cellulose treatment demonstrated a pronounced and extensive $^{14}CO_2$ release over the incubation period when exposed to the cellulose isolate, the cellulose substrate was selected for further examination as a parallel effort to that being conducted in the whole compost work.

Although isolates were obtained from whole compost and from compost fractions, none of these microorganisms were able to release substantial levels of $^{14}CO_2$ from the ^{14}C-TNT-labeled residues present in whole compost (Figure 4). Of the three groups of microorganisms, the group composed of isolates from the individual compost fractions produced the highest level of $^{14}CO_2$. However, all values of cumulative $^{14}CO_2$ were well below the 3-percent purity level, and neither the "fraction" nor the "whole compost" isolates showed $^{14}CO_2$ accumulations substantially greater that those observed for nonsterile control at Day 30 (Figure 4).

Fate of radiolabeled TNT-derived residues

Despite the fact that most of the ^{14}C-label in the whole compost was not mineralized to $^{14}CO_2$, a significant portion of the radiolabel accumulated in the glycolipid fraction. This suggests that microbial uptake and processing of the radiolabel from the ^{14}C-labeled compost may have incorporated the material into polyhydroxy alkanoates (PHAs, common bacterial storage (glyco)lipids] (Figure 5).

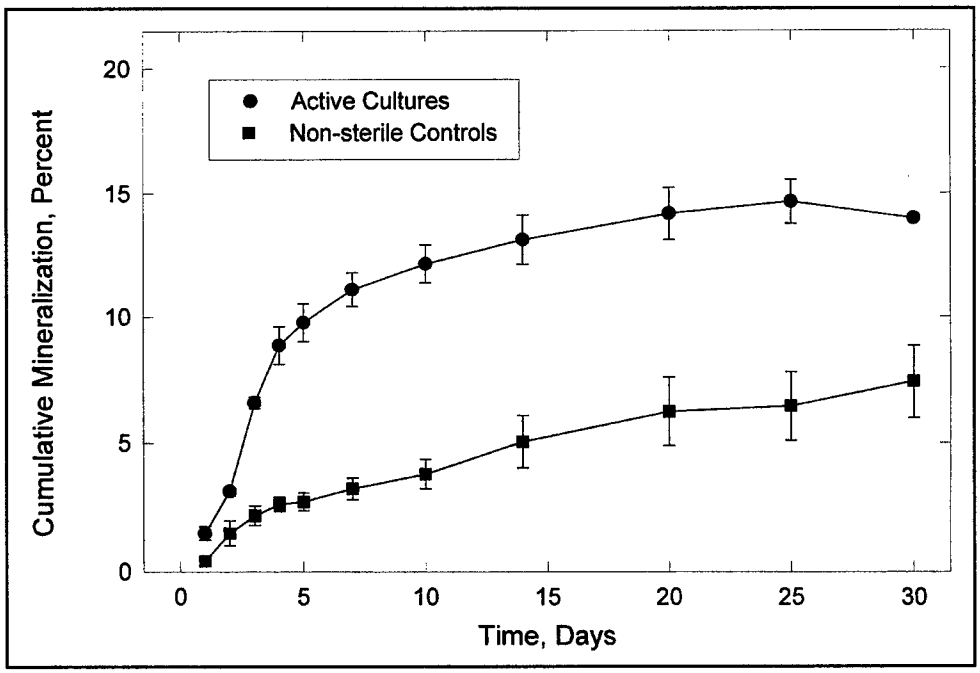

Figure 3. ^{14}C-TNT residue mineralization from fulvic acid fraction

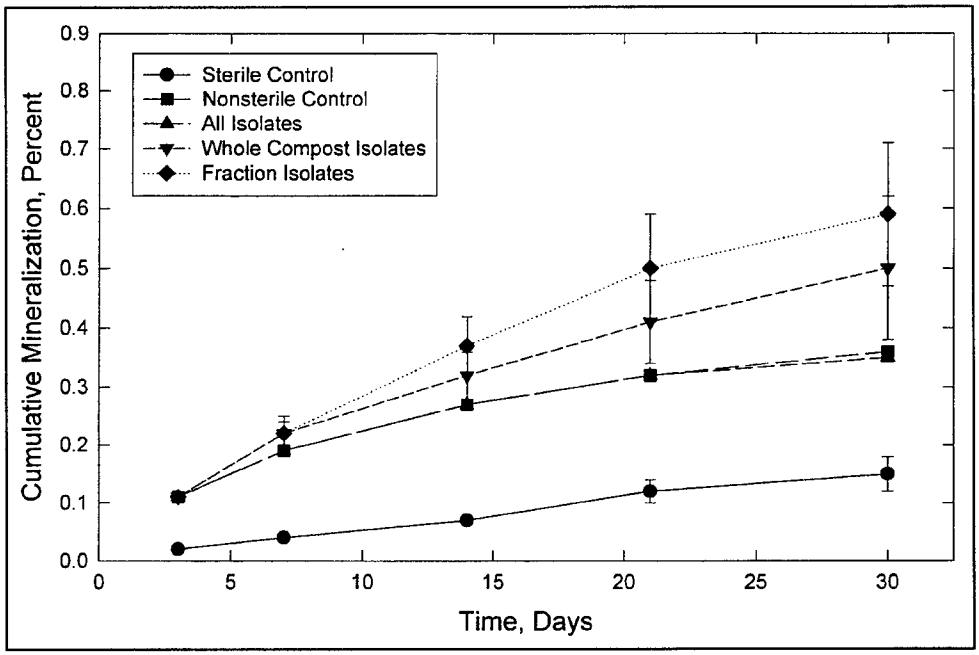

Figure 4. Mineralization of ^{14}C-TNT residue from amended whole compost

The potential for degradation and storage of the ^{14}C-label is supported by the fact that bacteria can store any carbon and energy source available when under nutrient limitations. The accumulation of PHA in the absence of cell division is termed "unbalanced growth" and can be represented by the ratio of polar lipid/ glycolipid

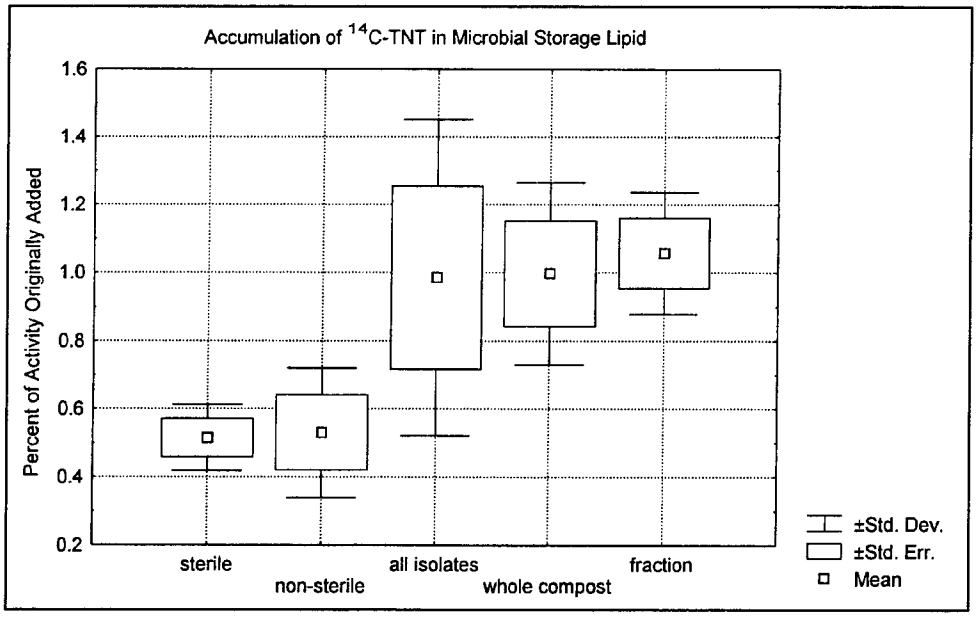

Figure 5. Accumulation of ^{14}C-TNT radiolabel from whole compost into microbial storage (glyco) lipids

(membrane lipid/storage lipid). No statistically significant decrease was observed in this ratio between any of the three groups and the sterile control (Figure 6). The total accumulation from the ^{14}C-conjugate into the bacterial glycolipid accounted for approximately 5 percent of the total radiolabel.

In contrast to whole compost, radiolabeled 14C-TNT residues in the cellulose fraction underwent extensive mineralization based on $^{14}CO_2$ evolution. At the end of the radioassay, the culture supernatants from the cellulose fraction were extracted and analyzed by HPLC for residual compounds in solution. Low levels of what were either explosives residues or soluble degradation products extractable as explosives residues, but with an undefined composition, were detected in the culture supernatants over time (Table 2). Material released from the cellulose incubation was also examined for the presence of TNT intermediates using HPLC with photo diode array detection. The resulting peaks were not identifiable as the parent compound or any known intermediates in aerobic or anaerobic TNT catabolic pathways, when compared with 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-2,6-dinitrotoluene (4ADNT), 2,4-diamino-6-nitrotoluene (2,4DANT), 2,6-diamino-4-nitrotoluene (2,6DANT), 2,4-dinitrotoluene (2,4DNT), 2,6-dinitrotoluene (2,6DNT), or either 2-, 3-, or 4-nitrotoluene standards. Total explosives concentration was, however, found to decrease over the 30-day incubation period, reaching background levels by Day 7 (Table 2). An attempt was made to identify the unknown peaks by use of electrospray and atmospheric chemical ionization (APCI) mass spectrometry. Results were inconclusive because of the inability to obtain concentrations of unknown analytes high enough to be detected by these procedures. Ion fragments obtained from the Day 3 material could not be related to any plausi ble TNT transformation product or known soil organic compound.

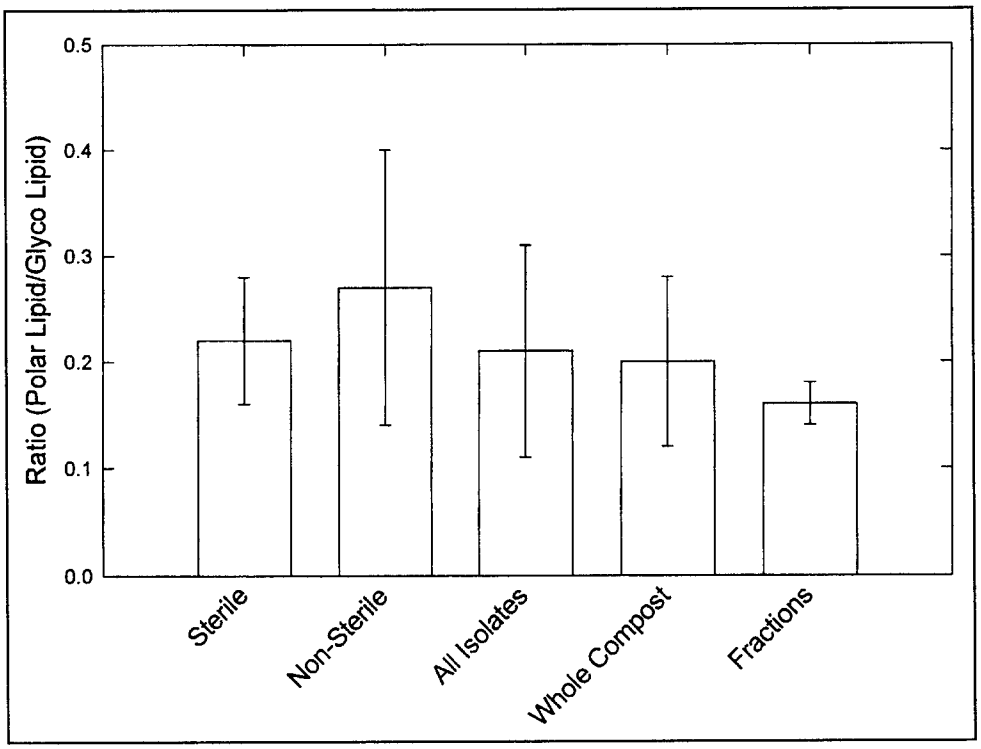

Figure 6. Estimation of unbalanced microbial growth on whole compost material

Table 2
Time Course Release of Compounds Unidentifiable by HPLC

Collection Time, days	Concentration, mg/L
0	0.46 ± 0.05
3	0.23 ± 0.03
7	0.07 ± 0.04
12	0.05 ± 0.06
17	0.06 ± 0.05
30	0.03 ± 0.04
Background	0.11 ± 0.01

Ability of whole compost and nutrient amendments to support TNT mineralization

The results of the radioassay with whole compost evolving $^{14}CO_2$ indicated that the conjugated residues were inaccessible to microbial attack. The *Burkholderia* isolate obtained from the cellulose fraction was able to release $^{14}CO_2$ from the cellulose fraction and also to survive and grow in the presence of whole compost. However, this microorganism was unable to release detectable levels of $^{14}CO_2$ from radiolabeled whole compost.

Neither the whole compost (WC) nor the cellulose (CELL) isolate mineralized uncomposted TNT to a significant extent. Addition of the nutrients NH_4Cl or K_2HPO_4 did not enhance the mineralization process (Figure 7). The microbial isolates in combination with the native microorganisms in nonsterilized whole compost did not attack the uncomposted [^{14}C]TNT. More than 98 percent of the freshly added radioactivity became bound to the solid phase. Approximately 11 to more than 21 percent of the radioactivity extracted from the solid phase was recovered in the organic and aqueous phases during the extraction process (Figure 8). Most of the extracted radioactivity in the organic phase was recovered in the semipolar fraction (glycolipids) (average of 4.23 ± 0.31 percent), while considerably less was found in the nonpolar lipid fraction (average of 1.43 ± 0.18 percent) and much less was found in the polar lipid fraction (average of 0.22 ± 0.02 percent) (Figure 9). Carbon is assimilated into membrane phospholipids during cell division (growth). In contrast, the carbon found in the glyco (storage) lipids is stored and then used for respiration. When the membrane lipid (PL)-to-storage lipid (GL) ratio was examined, the radioactivity was found in the storage lipids in the whole compost (WC) isolate and in the lipids from native microorganisms found in the biological control fractions (Figure 10). For the cellulose isolate, a higher portion of the radioactivity partitioned into the membrane phase. The amount of the radioactivity moving into the microbial cells was not impacted by the nitrogen or phosphate amendments (Figure 10). Thus, neither of these compounds is limiting the growth of any of the isolates. Readily available carbon is likely to be limiting in the whole compost treatment, but not in the cellulose fractions.

Microbial succession on compost and compost fractions

Analysis of microbial community composition on the basis of lipid fatty acid composition showed that none of the mixtures of isolates predominated after 30 days of incubation (Figure 11). Lipid biomarker profiles for the microorganisms present were not distinct for any of the treatments; however, some small differences among the treatments were apparent. When specific PLFAs were examined, the isolates introduced into the whole compost treatment retained some distinguishing traits throughout the 30-day incubation. The treatment containing the whole compost isolate showed significantly more biomass, while the other treatments produced biomass levels similar to those observed at time 0 (Figure 12).

In an incubation of the cellulose fraction with nonradiolabeled substrate over a 30-day period, a substantial change in community composition was measured (Figure 13). A loss in the lipids representative of gram-positive bacteria and a gain in those indicative of gram-negative bacteria were observed. The lipids exhibiting the greatest increase were prominent components of the cell membranes of the HA, ACN, and Cell isolates, all three of which are closely related based on lipid profiling. Increases in the relative percentage of the bacterial isolate recovered from the cellulose fraction correlated with the cellulose mineralization results described previously. These observations suggest that cellulose is conducive to the maintenance of a bacterial isolate capable of TNT mineralization.

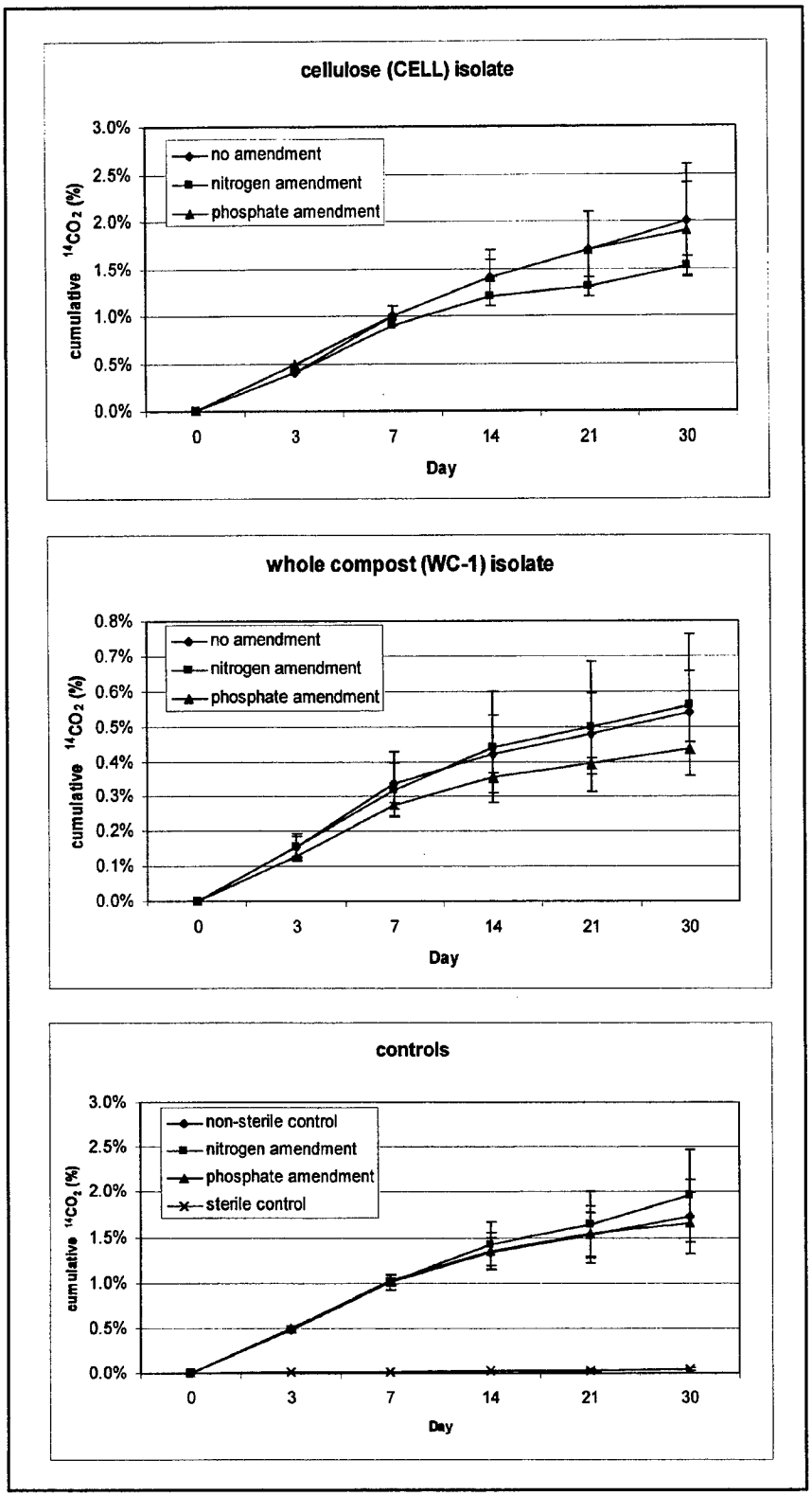

Figure 7. Impact of nitrogen and phosphate amendments on $^{14}CO_2$ evolution from whole compost incubated in presence and absence of CELL isolate and isolates WC (Nonsterile control contains native nicroflora, but no added isolates)

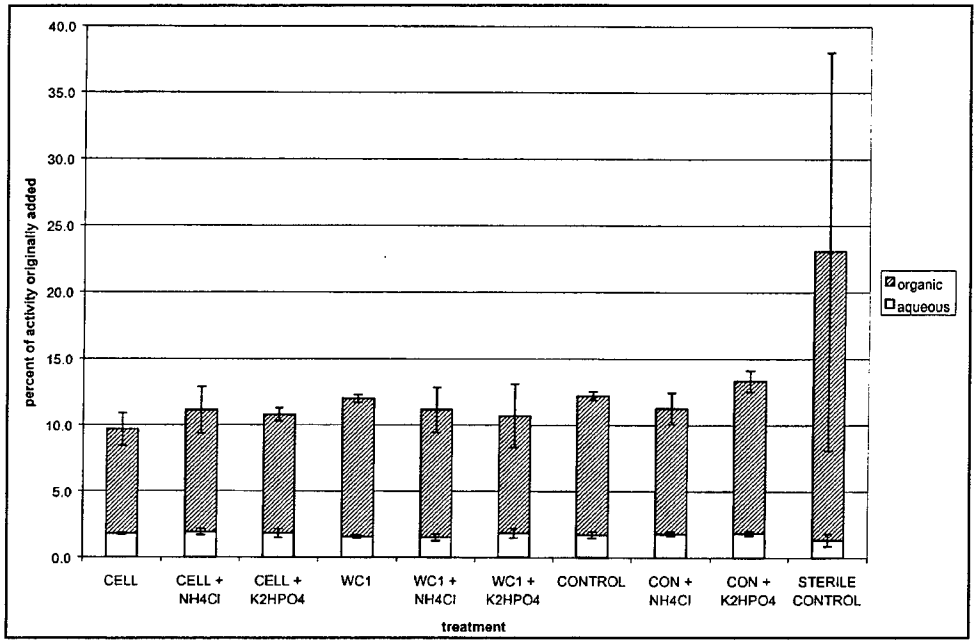

Figure 8. Partitioning of radiolabel from ^{14}C-TNT amendment into organic and aqueous phases (Initial activity was 4.0 x 10^5 DPM. Abbreviations: Cellulose = CELL; Whole compost = WC1; Biological control = CONTROL or CON)

Effects of pH and temperature on microbial activity

The isolates were capable of growing at all three pH values tested (5, 6, and 7), but the whole compost, humic acid, and cellulose isolate grew best at pH 6. Optimal pH for growth of the fulvic acid was pH 7, while the isolates from humin and ACN-extracts grew equally well at pHs 6 and 7.

The temperatures at which isolates are obtained from enrichment cultures are typically optimal for subsequent growth. However, the whole compost isolate, WC, and humic acid isolate, HA, barely grew at the 22 °C isolation temperature. The WC and HA isolates also grew poorly to moderately at 27 and 32 °C (Table 3). These results suggest that isolate WC and HA may have an optimum temperature above those used for these studies.

Carbon-14 mass balances

Very little of the added radioactivity was released as carbon dioxide or as carbonates (Table 4). Radioactivity in the culture supernatant was consistently between 1 and 1.2 percent; i.e., little water-soluble residual was evident at the end of the incubation period. About 60 percent of the radioactivity carbon was found in the solids phase. Thus, little degradation of the ^{14}C-TNT-derived residues in whole compost was evident.

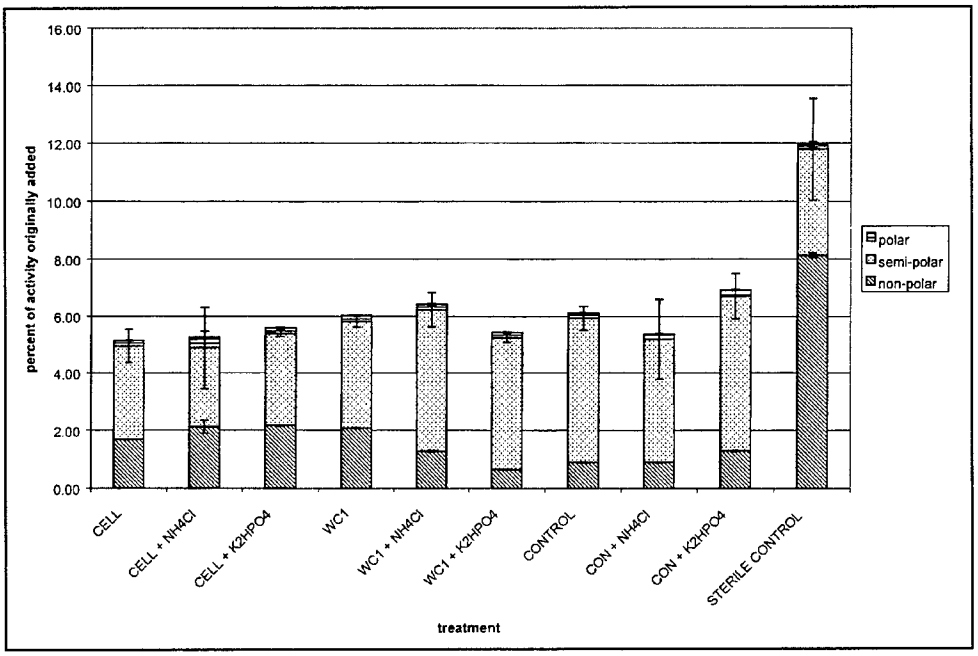

Figure 9. Partitioning of ^{14}C-TNT amendment into lipid classes (Initial activity was 4.0 x 10^5 DPM. Abbreviations: Cellulose = CELL; Whole compost = WC1; Biological control = CONTROL or CON)

Carbon-14 labeled mass balances for the cellulose fraction inoculated with the cellulose isolate indicate that more that 60 percent of the radioactivity remained with the culture at 30 days; approximately 23 percent was accumulated as $^{14}CO_2$, and less than 2 percent was left in the liquid phase (Table 5). The low residual in the liquid phase in combination with the loss from the solid phase and the high accumulation as carbon dioxide suggest that mineralization occurred in the liquid phase.

Mass balances conducted on the nonsterile controls at the end of the 30 days indicated that nearly 75 percent of the radioactivity was left in the solid phase. The radioactivity in the liquid and carbon dioxide phases (less than 10 percent) was nearly equal.

These results indicate that the cellulose fraction is degradable when separated from the whole compost. However, degradation of whole compost was limited, which suggests that disbursement of the cellulose in the whole compost limits availability of the substrate.

Other observations

The occurrence of conjugation reactions may very well explain the fate of TNT within the whole compost matrix and the subsequent inability of microorganisms to access the radiolabeled residues. Upon fractionation, much of the protective material (humic and fulvic acids, lignins, etc.) surrounding the radiolabeled-TNT residue is destroyed, and the structure of the residue may also be changed. These processes may explain the ability of the cellulose isolate to release $^{14}CO_2$ from the cellulose

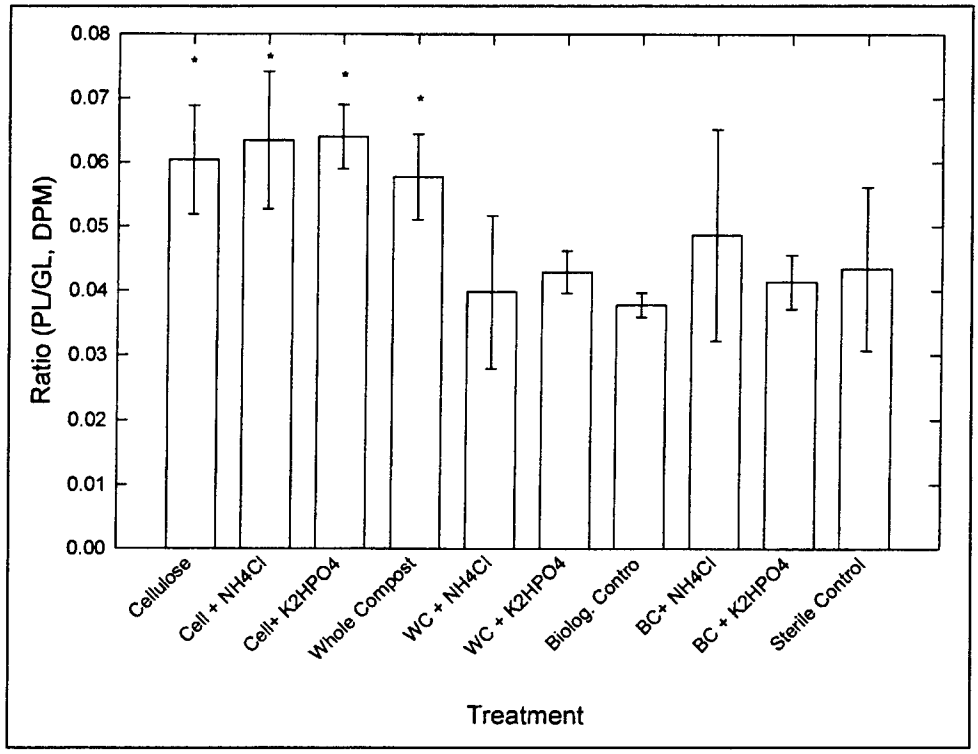

Figure 10. Estimation of unbalanced growth:partitioning of ^{14}C into membrane or storage lipids (Initial activity was 4.0 x 10^5 DPM. Abbreviations: Cellulose = Cell; Whole compost = WC; Biological control = BC. * = Significance at 95-percent level of confidence (Tukey HSD) versus control)

and (since the fulvic acid isolate appears to be the same microorganism) fulvic acid fractions. In addition, the use of a commercially available cellulase enzyme from the fungus *Trichoderma veridae* has shown that up to 50 percent of the cellulose fraction can be released as glucose, indicating that at least this much of the cellulose extract material is cellulose. The amount of glucose released from whole compost was approximately an order of magnitude lower than that for the cellulose. When either the cellulose fraction or whole compost containing ^{14}C-TNT-derived residues were digested with cellulase, however, no radioactive materials were released into solution. Therefore, the radioactivity was not associated with the cellulose per se. The chemical definition of the radiolabeled residue remains unknown.

Conclusions

Results of this study demonstrated that the composting process rendered TNT-derived residues unavailable to microbial attack. Very few microorganisms were able to grow using the whole compost as a carbon and energy source—i.e., the composting process consumes all available carbon sources, leaving only recalcitrant carbonaceous and/or nitrogenous materials. As a result, compost-bound TNT residuals will be released only very slowly, if at all, by microbial actions. The whole

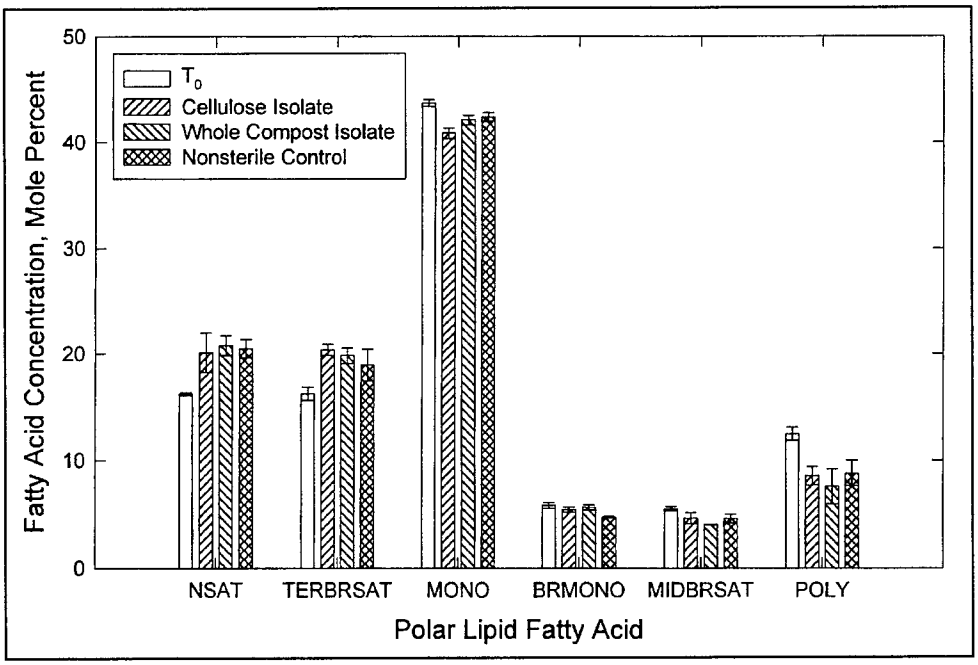

Figure 11. Changes in microbial community fatty acid composition on whole compost prior to and following 30 days of incubation with cellulose and whole compost isolates (Polar lipid fatty acid abbreviations: NSAT = Normal saturated; TERBRSAT = Terminally branched saturated; MONO = Monounsaturated; BRMONO = Branched monounsaturated; MIDBRSAT = Midchain branched saturated; POLY = Polyunsaturated)

compost to which the TNT residuals are attached is not likely to serve as a carbon and energy source for cometabolism. TNT residuals in the cellulose and fulvic acid fractions were readily degradable. Up to 23 percent of the radioactivity added as ^{14}C-TNT in the cellulose fraction and 15 percent in the fulvic acid fraction were released as $^{14}CO_2$ during a 1-month incubation period. Thus, the fractionation process apparently altered the bioavailability of the ^{14}C-TNT-derived residue or the structure to the point where cellulose and fulvic acid mineralization occurred. These results suggest that microbial activities will not release significant quantities of hazardous TNT derivatives from finished compost over time.

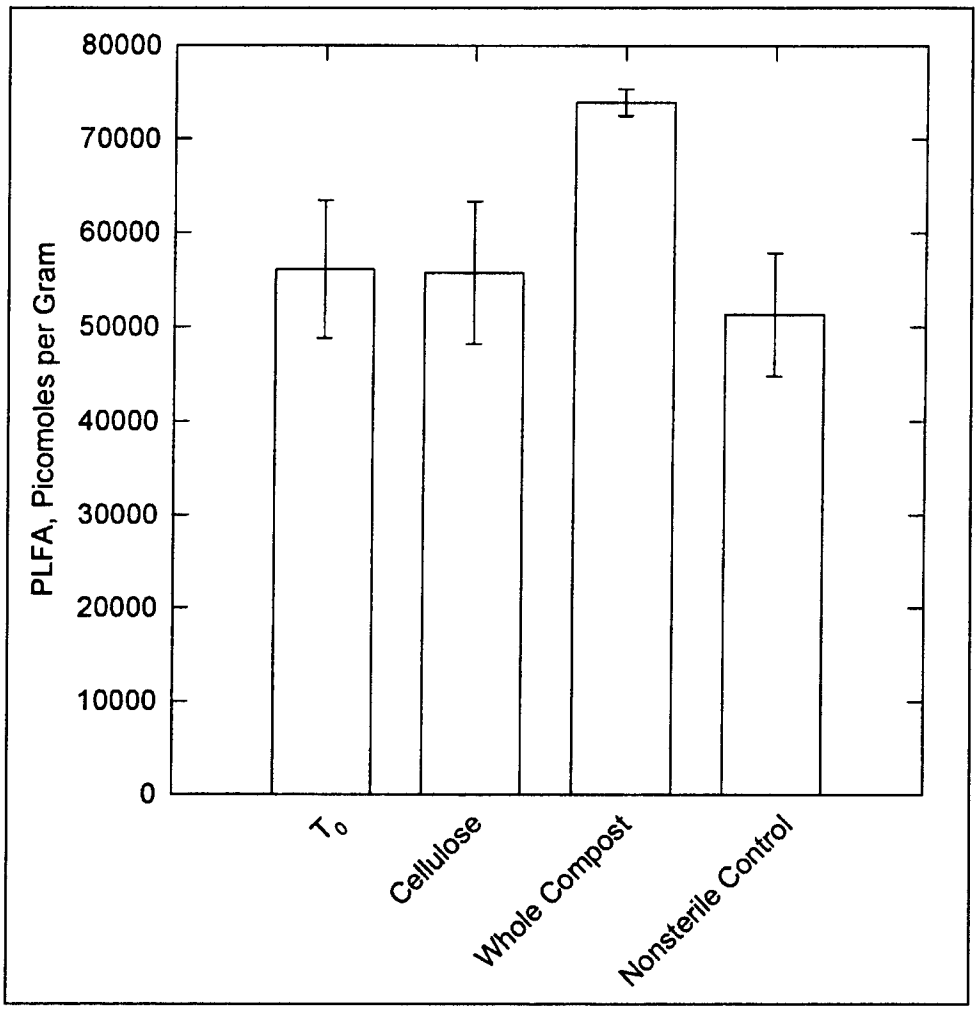

Figure 12. Microbial biomass estimates on whole compost at time 0 and on cellulose, whole compost, and the nonsterile control following 30 days

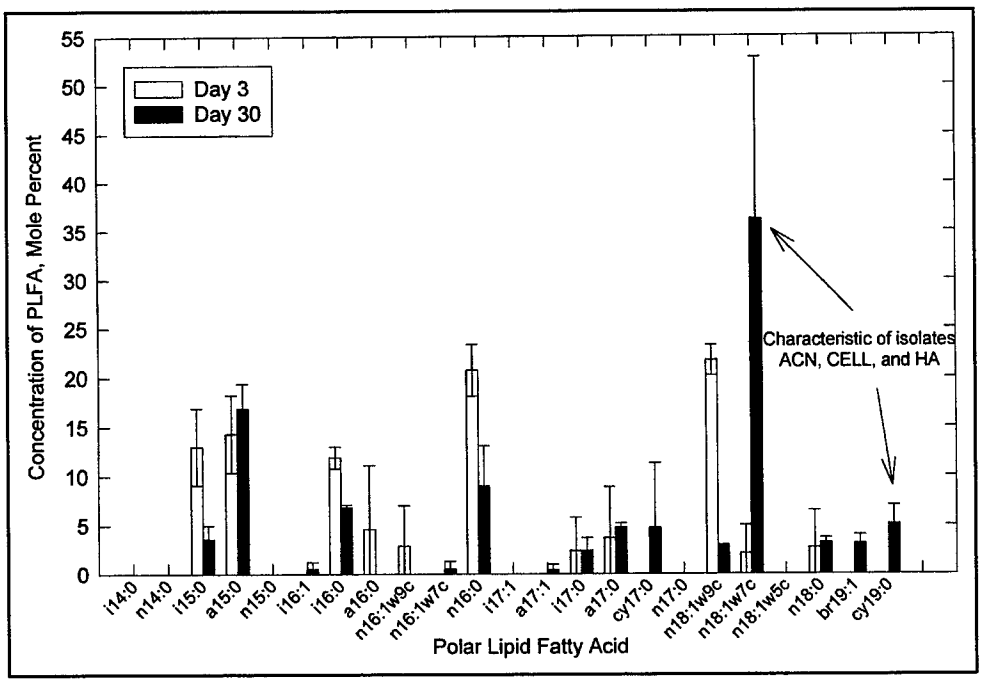

Figure 13. Changes in microbial community composition on cellulose prior to and following 30 days of incubation with cellulose and whole compost isolates (Abbreviations: Acetonitrile = ACN; Cellulose = CELL; Humic acid = HA)

Table 3
Effect of Temperature on Growth of Microbial Isolates[1]

Isolate and Abbreviation	22 °C	27 °C	32 °C
Cellulose (CELL)	+++	++	++
Whole compost (WC)	Wk	+	+
Fulvic acid (FA)	+++	+	+
Humin (Humin)	+++	++	++
Humic acid (HA)	Wk	+	+
Extractable (ACN)	+++	++	++

[1] Symbols: Wk, Less than 10% increase in growth; +, 10-35% increase in growth; ++, 36-75% increase in growth; +++, 76-100% increase in growth.

Table 4
Carbon-14 Mass Balances for Whole Compost Study (percent of added radioactivity)[1]

Inoculum	Solids ± S.E.	CO_2 ± S.E.	Carbonate ± S.E.	Liquid ± S.E.	Total Recovery ± S.E.
Sterile	60.1 ± 3.2	0.68 ± 0.52	0 ± 0	1.22 ± 0.13	64.9 ± 3.6
Nonsterile	63.4 ± 5.6	0.36 ± 0.01	0.11 ± 0.00	1.01 ± 0.03	64.9 ± 5.5
All[2]	65.9 ± 4.8	0.35 ± 0.00	0.08 ± 0.03	1.07 ± 0.01	67.5 ± 4.9
Whole compost	58.0 ± 2.1	0.50 ± 0.15	0.10 ± 0.06	1.10 ± 0.06	59.7 ± 2.0
Fractions[3]	63.3 ± 3.1	0.59 ± 0.15	0.14 ± 0.03	1.10 ± 0.08	65.1 ± 3.2

[1] Values presented are the means of three replicates ± the standard error of the mean (S.E.).
[2] Culture was nonsterile and was inoculated with the isolates from whole compost and compost fractions.
[3] Isolates from compost fractions only.

Table 5
Carbon-14 Mass Balances for Cellulose Fraction Study (percent of added radioactivity)[1]

Inoculum	Solids ± S.E.	CO_2 ± S.E.	Carbonate ± S.E.	Liquid ± S.E.	Total Recovery ± S.E.
Cellulose inoculated	67.8 ± 3.68	22.8 ± 0.91	0 ± 0	1.49 ± 0.10	92.1 ± 4.71
Nonsterile control	74.7 ± 2.64	6.91 ± 0.05	0 ± 0	7.09 ± 0.44	88.7 ± 3.13

[1] Values presented are the means of three replicates ± the standard error of the mean (S.E.).

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5 Toxicity of TNT, RDX, and HMX to Earthworms, *Eisenia foetida*, in Soil and Finished Compost

Introduction

Although the toxicity of TNT and its potency as a mutagen in bacterial assays is well recognized, and composting has been shown to attenuate these effects remarkably in soil (Tan et al. 1992; Griest et al. 1993, 1995), significant acute toxicity may still be retained in a finished compost, and bacterial mutagenicity may be substantial (Jarvis, McFarland, and Honeycutt 1998). Additionally, even in a compost in which over 90 percent of the TNT present in the precompost soil has disappeared, as much as 25 percent of the original concentration has been recovered using acidic extraction (Breitung et al. 1996).

Most studies on the efficacy of composting for remediation of explosives contamination have relied on analytical chemistry to quantify the results. Few have also included toxicity, and nearly all of those have measured only acute toxicity and bacterial mutagenicity, often in aquatic organisms, not chronic sublethal effects on terrestrial organisms. From an ecological point of view, the death of individual organisms is far less consequential than are chronic effects such as impaired growth or reproduction. The latter have the potential for adverse impacts at population or community levels. Additionally, very few studies have been performed on the toxicities of RDX or HMX that were not confounded by the presence of TNT. Although the acute toxicity of either of these compounds is considered to be low, nothing is known of their chronic effects.

The purpose of the investigations reported here is to more fully characterize the toxic nature of TNT, HMX, and RDX in soil and composted soil by using long-term exposures of the common earthworm, *Eisenia foetida*, an ecologically important organism that would normally be expected to encounter explosives were they present in its habitat. The effects of the compounds as individuals were evaluated by spiking the single chemicals into a natural soil. A suite of assays rather than one

or two unrelatable end points was used in order to maximize the information gained and provide insight into the modes of toxic action of the explosives.

Materials and Methods

Acute toxicity of TNT

Artificial soil (AS) composed of 70-percent industrial sand, 20-percent kaolin clay, 10-percent peat moss, with calcium carbonate added to adjust the pH to 6.5-7.5 and moisture content adjusted to 50 percent (Greene et al. 1989) was spiked at a nominal concentration of 2,000 mg TNT kg^{-1}. Serial dilutions were made with equal weights of additional AS to provide a concentration range of 2.0-2,000 mg TNT kg^{-1}. Five replicate exposures were made at each concen tration and were prepared according to the method of Gibbs, Wicker, and Stewart (1996). Each replicate consisted of a pair of adult earthworms with 100 g of the soil placed in a perforated Ziploc sandwich bag. The bags were held at room temperature (approximately 20 °C) under constant illumination (ambient room fluorescent). After 14 days, the bags were opened and survivors counted. The LC_{50} was then calculated for the TNT exposures using the trimmed Spearman-Karber method (EMSL 1993).

Preparation of the soils and composts

Soil was collected from the Longhorn Army Ammunition Plant (LHAAP), Karnack, TX. Chemical analysis showed that the soil contained 0.5 ppm TNT, and <0.1 ppm of RDX or HMX or of any degradation product of TNT. A 700-g aliquot of the LHAAP soil was spiked at approximately 2 ,000 mg kg^{-1} with TNT. Chemical analysis subsequently showed the concentration of TNT to be 1,855 mg kg^{-1} with 3.13 mg 1,3,5-trinitrobenzene kg^{-1}, 1.62 mg 2-amino-4,6-dinitrotoluene kg^{-1} and 1.08 mg 2,4-dinitrotoluene kg^{-1} (Table 1). The TNT-spiked stock was then diluted tenfold (w:w) with AS to obtain the TNT-spiked soil at the targeted concentration for chronic exposures (approximately 200 mg kg^{-1}, dry weight). The TNT-spiked stock was also diluted tenfold (w:w) with composting amendments (Table 2) to obtain precompost TNT-spiked soil (Figure 1). Unspiked LHAAP soil was similarly diluted with artificial soil to provide the laboratory control (control soil) and with composting amendments to provide precompost control soil (Figure 1). The earthworms were originally intended to be exposed to three mixtures: (a) the TNT-spiked soil, (b) the precompost TNT-spiked soil, and (c) the TNT finished compost. However, the precompost TNT-spiked soil was lethal to the earthworms; therefore, further experiments were not conducted with this soil.

RDX- and HMX-spiked stocks were prepared in the same manner, but at 5,000 mg kg^{-1}. Chemical analysis showed the achieved concentrations to be 4,230 mg HMX kg^{-1}, and 3,970 mg RDX kg^{-1} (with 371 mg HMX kg^{-1} in the RDX as an adulterant). Fresh control soils were prepared at the time the RDX- and

Table 1
Explosives Concentrations in TNT-, RDX-, and HMX-Spiked Stock and Finished Compost, mg kg^{-1}

Treatment	Analyte									
	TNT	RDX	HMX	TNB[a]	DNB[b]	Tetryl	4ADNT[c]	2ADNT[d]	2,6DNT[e]	2,4DNT[f]
TNT-spiked stock	1,855	<0.100	<0.200	3.13	<0.100	<0.100	<0.100	1.62	<0.100	1.08
RDX-spiked stock	<0.100	4,020	371	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100
HMX-spiked stock	<0.100	3.52	4,165	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100
TNT finished compost	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100	4.55	<0.100	<0.100	<0.100
RDX finished compost	<0.100	418	50.9	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100
HMX finished compost	<0.100	<0.100	1,086	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100	<0.100

[a] 1,3,5-trinitrobenzene.
[b] 1,3-dinitrobenzene.
[c] 4-amino-2,6-dinitrotoluene.
[d] 2-amino-4,6-dinitrotoluene.
[e] 2,6-dinitrotoluene.
[f] 2,4-dinitrotoluene

Table 2
Composition of Composts

Component (function)	Percentage of mixture	
	Dry weight	Wet weight
LHAAP soil (contaminated or control)	10.0	
Sawdust (bulking agent)	19.8	
Apple pomace (organic carbon)	5.4	
Chopped potato (organic carbon)	15.3	
Alfalfa (organic carbon)	19.8	
Green cow manure (inoculum)	29.7	
Water		60.0

HMX-spiked stocks were diluted with artificial soil or with composting amendments.

One-half of the precompost TNT-spiked soil and one-half of the precompost control soil were composted in two 40-L adiabatic reactors (Oxynax Reactor, Columbus Instruments, Columbus, OH). After 8 days, the temperature had decreased to ambient levels, and the composting was terminated after 16 days (Figure 2). The same proportions of soil and amendments were used in the RDX and HMX experiments, and the temperature profiles similarly fell to ambient levels

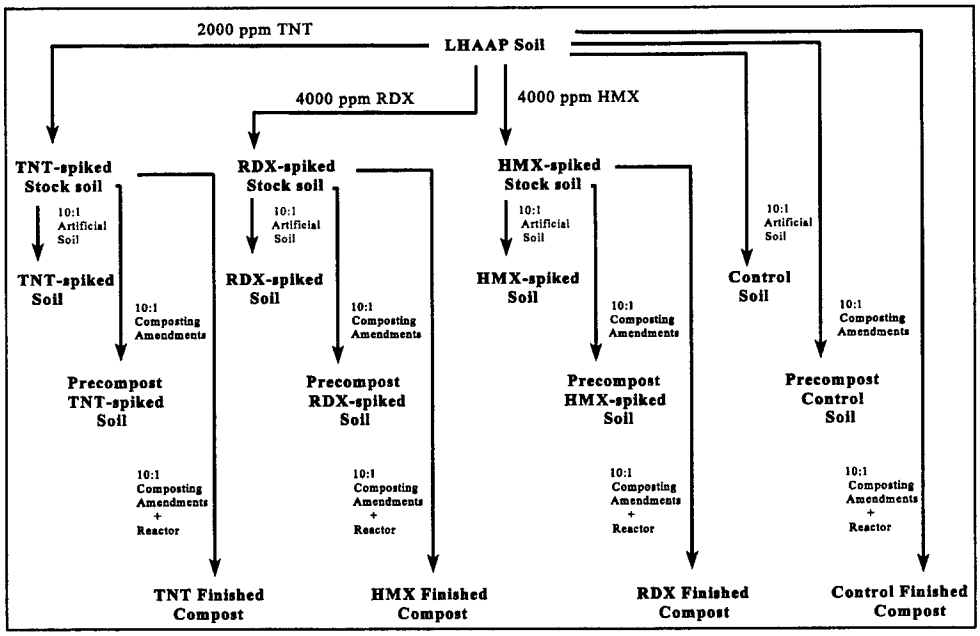

Figure 1. Preparation of experimental and control soils

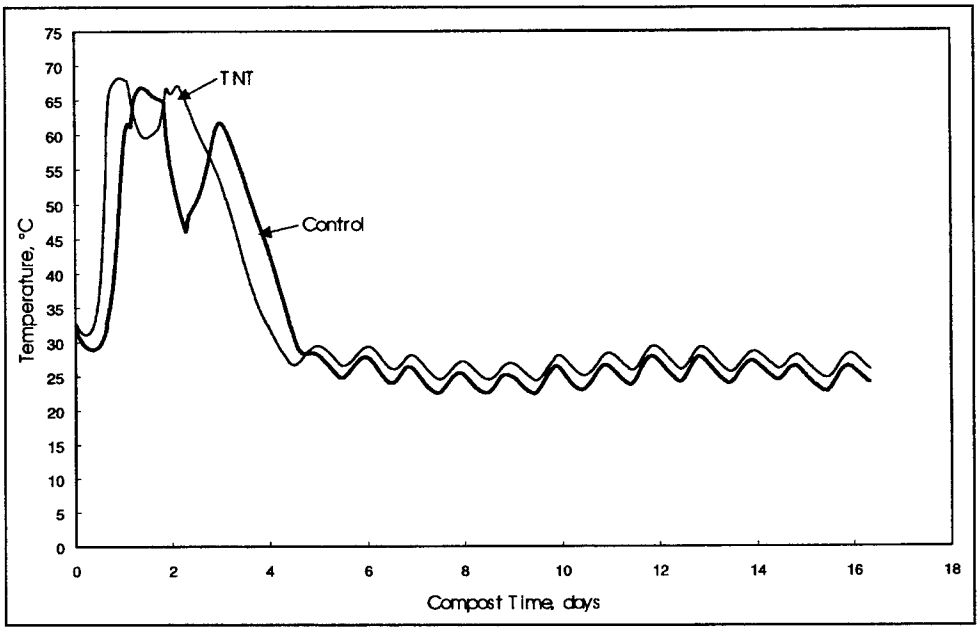

Figure 2. Temperature profiles of TNT-spiked soil and control soil composts

after about 8 days (Figures 3-4). Composting of the RDX- and HMX-spiked soils was continued for an additional 20 days.

Experimental organisms

Earthworms, *E. foetida*, were maintained in continuous culture from stocks obtained from Carolina Biological Supply Company (Burlington, NC). Worms were kept in moistened sphagnum peat and fed *ad lib* on a diet of Magic Worm Food (Carolina Biological Supply Co.) Young adults weighing 0.25-0.40 g live weight were selected for all experiments.

Survival, growth, and reproduction exposures

Survival, growth, and reproduction exposures were conducted following the methods of Gibbs, Wicker, and Stewart (1996). Earthworms were exposed in perforated Ziploc bags containing 100 g of TNT-, HMX-, or RDX-spiked soil, TNT finished compost, control soil, or control finished compost. Twenty bags were prepared for each treatment. Exposure conditions were as described for the TNT acute toxicity test. Worm pairs were preweighed before being placed into the bags, and surviving worm pairs were weighed again after 21 days to deter mine growth. Cocoons were also counted at that time as the measure of reproduction.

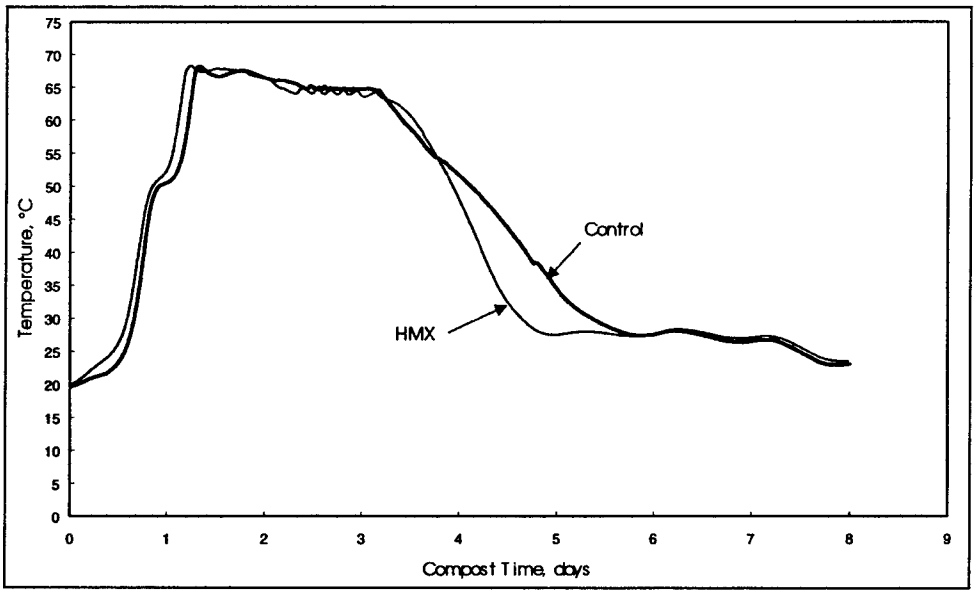

Figure 3. Temperature profiles of HMX-spiked soil and control soil

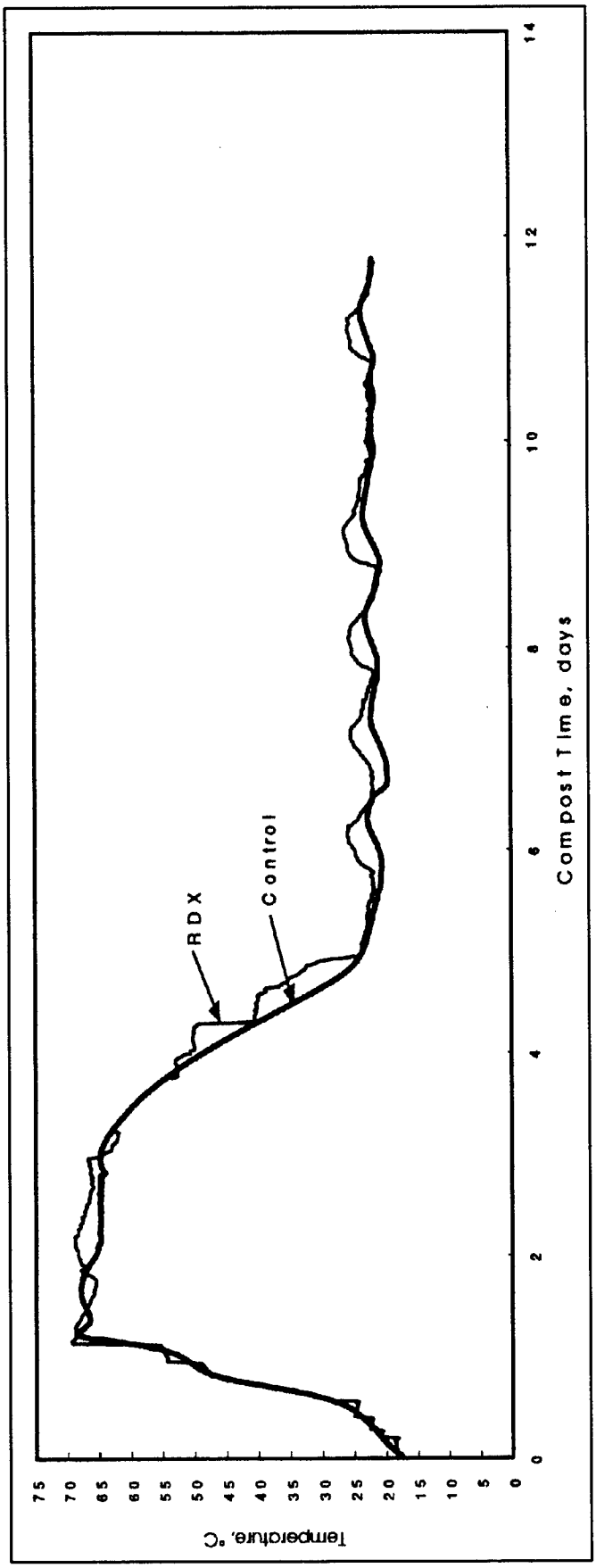

Figure 4. Temperature profiles of RDX-spiked soil and control soil composts

Exposures for biomarker assays

An additional set of 20 bags for each treatment containing two worms each were prepared similarly to those for the survival, growth, and reproduction assays. After 21 days, the worm pairs were rinsed with a saline solution, placed in cryovials, frozen in liquid nitrogen, and transferred to a -80 °C freezer for storage. Half of the samples were analyzed for single strand DNA (ssDNA) breaks and the other half for enzyme activity. Enzyme assays included superoxide dismutase (SOD), catalase (CAT), both selenium dependent (GPX/HP) and selenium independent (GPX/CP) glutathione peroxidase, glutathione reductase (GR), and glutathione-S-transferase (GST). Postmitochondrial fractions were used for SOD and GPX analysis, while cytosolic fractions were used for CAT, GR, and GST. Coelomocytes were non-invasively extruded from worms from a third set of 20 bags for use in the immuno-assay, after which the worms were placed in cryovials, frozen in liquid nitrogen, and stored in a -80 °C freezer until analysis for glutathione.

Avoidance tests

Avoidance tests were conducted by the methods of Yeardly, Lazorchak, and Gast (1996). Circular glass culture dishes (200 by 80 mm) were divided into halves using a cardboard strip; artificial soil was placed in one side and the test medium in the other. The divider was then removed, and 10 worms were placed on the dividing line. After 4 days, the two soils were separated along the divid ing line, and worms were counted on each side. In addition to the TNT-spiked soil (approximately 200 ppm) and the control soil, positive and negative controls (KCl-spiked soil and artificial soil, respectively) were tested; five replicates were conducted for each treatment. An experiment was also run to test the effect of composting on avoidance behavior. The dishes were prepared with one side containing control finished compost and the other containing TNT finished com post. The RDX and HMX avoidance assays were performed together and shared the same control soil.

Enzyme assays

Samples were homogenized 1:4 (w:v) in buffer (250 mM sucrose, 80 mM Tris HCl, 5 mM $MgCl_2$, 250 mM KCl, 1 mM EDTA, pH 7.4) using a motor-driven ground glass pestle with glass homogenizing tube. Triton X-100 was added after homogenization to obtain a final concentration of 0.5 percent Triton X-100 and the sample sonicated (setting 2, two 10-s bursts). Samples were then centrifuged at 9K × g for 15 min. For SOD and GPX assays, the supernatant was retained as the postmitochondrial (S9) fraction. For the remaining assays, the supernatant was transferred to a clean tube and centrifuged a second time at 100K × g for 1 hr at 4 °C, and the supernatant was retained as the cytosolic fraction. Protein concentrations were determined using the BioRad protein assay (96-well plate format) with bovine serum albumin as the standard; samples were diluted in triplicate for protein analysis.

The SOD assay was conducted as described in Mishra and Mishra (1996). Superoxide was generated by a xanthine-xanthine oxidase system, and the amount formed was monitored at 550 nm by the rate of cytochrome c reduction. A standard SOD unit was defined as the amount of protein required to inhibit the reaction rate by 50 percent. GPX/HP and GPX/CP were measured using either H_2O_2 or cumene hydroperoxide as substrates and monitoring the loss of NADPH as the reaction progressed (Flohe and Gunzler 1984). The assay mixture (50 mM phosphate buffer, pH 7.6 with 0.1 mM EDTA, 0.15 mM sodium azide, 3.0 mM GSH, 0.25 mM NADPH, and 0.67 U/ml GR) was kept at 28 °C in a water bath and used within 4 hr of preparation. Six wells of the 96-well plate were coated with 20 µL homogenizing buffer to determine the background rate of reaction, and the remaining wells were coated with 10 or 20 µL of each sample (in triplicate). Homogenizing buffer was added to bring the final sample volume in each well to 20 µL. The assay mixture (200 µL per well) was added and the plate preincubated in a 28 °C incubator for 2 min, after which the reaction was initiated by adding 50 µL of substrate (1 mM cumene hydroperoxide for 0.2 mM final assay concentration, or 1.25 mM H_2O_2 for 0.25 mM final assay concentration). Absorbance was read at 30-s intervals at 340 nm for 3 min at room temperature, and activity was linear with respect to sample volume for at least 0.030 OD/min with H_2O_2 as a substrate (selenium dependent GPX) and at least 0.025 OD/min with cumene hydroperoxide as a substrate (selenium-independent GPX). Activity was reported as nmol NADPH consumed/min*mg (E = 6.22 cm^2/µmole for NADPH, b = 0.8 cm, volume = 0.26 ml).

The CAT assay was conducted as described by Cohen, Mimi, and Vivian (1996) and used cytosolic fraction. Loss of H_2O_2 was monitored indirectly by reaction with Fe^{2+} ions to form Fe^{3+} ions, followed by reaction of Fe^{3+} with thiocyanate to form the reddish ferrithiocyanate complex (absorbance maximum at 450 to 480 nm). Activity was reported as rate constant '*k*', where $k = (\ln A_1/A_2)/[(t_2-t_1)*$ mg cytosolic protein] where A_1 is absorbance at 460 nm time 1 (t_1) and A_2 is absorbance at 460 nm at time 2 (t_2). Cytosolic samples were diluted to the point that the change in absorbance was less than 0.5 AU for t_1= 1 min and t_2= 4 min (1:10 dilution).

Cytosolic samples were used without dilution for the GR assay. The method of Cohen and Duvel (1988) was adapted to the 96-well plate format. The assay mixture (preheated to 25 °C prior to the assay) contained 20 ml buffer (sodium phosphate buffer, 0.2 M, pH 7.6), 2.5 ml GSSG (10 mM in buffer), and 2.5 ml NADPH (1 mM in buffer) for a final concentration of 1 mM GSSG and 0.1 mM NADPH. Six wells of the 96-well plate were coated with 20 µL homogenizing buffer to determine the background rate of reaction, and the remaining wells were coated with 10 or 20 µL sample per well in triplicate; homogenizing buffer was added to bring the final volume in each well to 20 µL. The reaction was initiated by addition of 0.2 ml of the assay mixture per well, and NADPH loss was recorded over 10 min (absorbance at 340 nm; read at 2-min intervals; plate returned to the incubator between readings). The plate was read at 340 nm every minute for 10 min at 25 °C. Background corrected values did not exceed -10 mOD/min in order to retain linear rates. Activity was reported as nmol NADPH consumed/min*mg (E = 6.22 cm^2/µmole for NADPH, b = 0.6 cm, volume = 0.22 ml).

The GST assay was adapted to the 96-well plate format from methods of Habig and Jakoby (1981). Samples were diluted with homogenizing buffer to obtain a rate of reaction of less than 0.02 OD/min to maintain linearity of the reaction (between 10 and 30 µL in 1 ml homogenizing buffer). The assay reac tion mixture was prepared by adding 0.5 ml of 42 mM CDNB (in ethanol) to 20 ml of 0.2 M phosphate buffer (pH 6.5). The first 6 wells of the 96-well plate were coated with 10 µL buffer to determine the background rate of reaction, and the remaining wells were then coated with 10 µL diluted sample, 3 wells per sample. After the plate was coated, 0.5 ml of 42 mM GSH was added to the assay reaction mixture, vortexed, and 200 µL of the mixture immediately added to each well on the plate using an eight-channel pipetter. Absorbance was monitored at 30-sec intervals at 340 nm for 3 min to obtain the change in absorbance/minute. The rate of conjugation of CDNB was divided by the amount of protein, and units were expressed as µmol/min*mg (E = 0.96 cm^2/µmole , b = 0.6 cm, volume = 0.22 ml).

Glutathione

Aliquots were homogenized in 5-percent S- sulfosalicylic acid (SSA) using a Brinkman polytron homogenizer (0.1-0.2 g of sample per ml of SSA), then centrifuged for 10 min at 13K x g after sitting on ice for 15 min. The resulting supernatant was then diluted fortyfold to fiftyfold in phosphate buffer (0.2 M phosphate buffer, pH 7.5) for TGSH analysis. Total glutathione was measured using the 96-well plate method of Baker, Cerniglia, and Zaman (1990). GSSG standards were prepared over a 0.05- to 2.0-ng/ml range and the plate coated in triplicate with 50 µL of the standards. The assay cocktail was prepared by adding 2.8 ml of 1 mM 5,5'-dithiobis(2-nitrobenzene), 3.75 ml of 1 mM NADPH, and 5.85 ml of 0.2 M phosphate buffer (pH 7.5). Just before reading the plate, 20 units of GR were added to the cocktail, and 100 µL of this mixture immediately placed in each well using an eight-channel pipette. The plate was read every 15 sec at 405 nm for 2 min. Results (mg TGSH/ml) were corrected for dilution factors, converted to nmol TGSH, and divided by the tissue weight to obtain nmol TGSH/g tissue.

For the GSSG assay, a second dilution of the supernatant of the centrifuged homogenate was made. The sample was diluted twofold to fivefold with phos phate buffer, and the pH of a 0.5-ml aliquot was adjusted to 7.0 with triethanolamine before the addition of 5 µL of 2-vinylpyridine. Standards were prepared by adding 5 µL of 2-vinylpyridine to 0.5 ml of GSSG standards (0.05- to 2.0 -ng/ml range). Samples and standards were covered with foil and placed in a dark hood at room temperature for at least 1 hr to derivatize any GSH, then were analyzed in the same manner as described for TGSH.

Immunotoxicity

The immunotoxicity tests were conducted as described by Chen et al. (1991) and included total number of extruded coelomocytes, live versus dead cells, and a nitroblue tetrazolium dye (NBT) reduction assay. Briefly, coelomocytes were collected from the worms by placing worm pairs into 4 ml of extrusion formula

(5-percent ethanol in 8.5 mg/ml NaCl , 2.5 mg/ml EDTA, and 10 mg/ml guaiacol glycerol ether, pH 7.3) for 2 min. The extruded coelomocytes were then rinsed twice with calcium-free Hanks buffered saline solution (HBSS). The extruded coelomocytes from two worm pairs were combined and suspended in 1 ml of HBSS. The total number of cells extruded, and the number of live versus dead cells were determined utilizing a hemocytometer and trypan blue to distinguish live cells from dead cells. Based on the total cell count, the volume of coelomocytes required for 1 $\times 10^6$ cells was placed in a siliconized glass tube along with 0.1 ml of 0.01 M potassium cyanide and 0.05 ml of a nonviable bacterial extract (STIMULANT; Sigma Chemical Co.). This mixture was gently shaken and incubated for 15 min at 25 °C before adding 0.1 ml of NBT (0.56 mM in 0.1 M KPO_4 buffer, pH 7.8, with 0.1 mM EDTA). The tubes were then incubated at 25 °C for 1 hr before stopping the reaction with the addition of 4 ml 0.5 M HCl. The tubes were then centrifuged (1000 × g for 10 min), the supernatant discarded, and the NBT extracted from the cells by the addition of 1.5 ml of pyridine into the tube and incubating it in boiling water for 10 min. After allowing the sample to cool, the absorbance at 515 nm was determined.

DNA damage

Unless otherwise noted, all procedures for ssDNA breaks were conducted on ice. Tissue aliquots were homogenized by hand in 1 N NH_4OH with a 5-ml ground glass homogenizing tube; the minimum number of strokes to complete homogenization was used. The samples were transferred to a Teflon centrifuge tube with 2 ml water and 6 ml chloroform-isoamyl alcohol-buffered phenol (24:1:25, v:v:v) added. The samples were gently inverted 10 times to denature proteins and allowed to sit for 10 min. The aqueous phase was transferred to a new Teflon centrifuge tube, rinsed twice with ice-cold chloroform, and centri fuged at 5K × g for 10 min followed by a second centrifugation at 13K × g for 15 min. DNA was precipitated from the resulting supernatant by adding 1/10th volume of 0 .2 M sodium acetate and 2.5 × volume of ethanol, then storing the sample in the freezer for at least 1 hr. The sample was then centrifuged (800 x g for 10 min), the pellet rinsed first with 70-percent ethanol then with ethyl ether, and was then dissolved in 3 to 5 ml buffer (150 mM NaCl, 10 mM Tris HCl, 1 mM $MgCl_2$, and 0.5 mM EDTA, pH 7.4) to give an initial fluorescence with Hoechst dye of approximately 100 FU.

To determine the initial or double-stranded DNA (dsDNA) fluorescence, 75 µL of 0.05 M NaOH, 75 µL of 0.05 M HCl, and 10 µL of 0.2 percent SDS in 2 mM EDTA were placed in an amber vial and mixed before adding 0.25 ml of the DNA sample. The sample was forced through a 20-gauge needle five to seven times, and 1 ml of 0.2 M phosphate buffer (pH 6.9) was added, followed by 1.5 µL Hoechst 33258 dye (1 mg/ml). The sample was mixed and allowed to sit in the dark for 15 min to allow fluorescence to stabilize before the fluores cence was monitored at 360 nm excitation and 450 nm emission. The fluores cence of single-stranded DNA (ssDNA) was determined by adding 75 µL of 0.05 M NaOH to 0.25 ml of sample and incubating for 90 min at 80 °C. After incubation, the sample was neutralized by addition of 75 µL of 0.05 N HCl and 10 µL of 0.2-percent SDS in 2 mM EDTA, then forced through the needle before addition of 1 ml of the phosphate buffer and 1.5 µL of the Hoechst dye. The alkaline unwound DNA (AU DNA) was treated in a similar manner, except that the samples were incubated for 80 min at 45 °C before

neutralization. Each sample was run in duplicate for dsDNA, ssDNA, and AU DNA, and the F-value calculated utilizing the average fluorescence obtained from the duplicates (F-value = (AU DNA-ssDNA)/(dsDNA-ssDNA)).

Statistical analysis

Data were subjected to analysis of variance after first testing assumptions of normality using Shapiro-Wilk's test and equality of variances using Levene's test. In most cases, assumptions were met using untransformed data, but in some instances conversion to rankits (normalized ranks) or square root transfor mation was necessary (SAS 1988). One-way analysis of variance was used, and means were compared by Tukey's test and by Fisher's Least Significant Differ ence (LSD) when more than two parameters required comparison. Student's t -test was used to compare means of the glutathione assays in the TNT-spiked soil exposure. A one-tailed binomial test was used in the avoidance bioassay.

Results and Discussion

Acute toxicity

The results of the TNT acute toxicity test were used to establish the exposure concentration used in all subsequent TNT chronic toxicity experiments. The 14 -day LC_{50} to *E. foetida* of TNT in LHAAP soil serially diluted with AS was 353 mg kg^{-1} with a 95-percent confidence interval of 304 to 410 mg kg^{-1}. A concentration of 200 mg kg^{-1} was considered to be at or below the lethal threshold and was selected as the target concentration for preparation of the TNT-spiked soil and precompost TNT-spiked soil for chronic exposures (Figure 1).

An attempt was made to similarly find 14-day LC_{50}s for RDX and for HMX. However, no mortality was observed at approximately 4 ,000 mg kg^{-1} of RDX or of HMX in spiked LHAAP soil after 14 days. During the acute toxicity exposures, a nonquantified aversive coiling response was observed. The coiling response attenuated with AS dilution, but persisted down to about 12.5 percent of the spiked LHAAP soil. The coiling response was absent below 12.5 percent. The exposures were continued for an additional 7 days at which time they were terminated, still with no mortality. The conclusion was that HMX and RDX in soil are not acutely toxic to earthworms. Therefore, because approximately 4,000 mg kg^{-1} was the highest concentration that could practicably be spiked into the LHAAP soil with a homogeneous distribution, that was the concentra tion used in the RDX and the HMX stocks (Figure 1). All chronic exposures of earthworms to these compounds were conducted at approximately 400 mg kg^{-1} with these compounds.

Chronic toxicity

No worms in the TNT-spiked soil or in the control soil died during the chronic exposures, but a loss in tissue dry weight indicated a negative effect on growth in both treatments (Table 3). No cocoons or juveniles were produced in either soil, indicating a negative effect on reproduction. Because the effects were similar in both the control soil and the TNT-spiked soil exposures, appar ently nontreatment stress rather than TNT toxicity was the cause of the negative growth and the reproductive failure. In contrast, toxicity of the TNT finished compost was very high with only 35 percent of the worms surviving to the eighth day of exposure, whereas 96 percent of the earthworms in the control finished compost were alive at 21 days. The toxicity was not directly attributable to TNT, as chemical analysis of the finished compost showed <0.1 ppm extractable TNT remained. However, extractable 4-amino-2,6-dinitrotoluene (4ADNT) was present at a concentration of 4.55 mg kg^{-1}. Metabolites of TNT rather than the parent compound are likely to be the agents of acute toxicity in the finished compost. Survivors were insufficient in numbers in the TNT finished compost to statistically compare growth with that in the control finished compost. However, weight of earthworms in the control finished compost increased. Reproduction was zero in both composted treatments; i.e., no cocoons or juvenile worms were found.

Table 3
Survival and Growth of Earthworms in TNT-Spiked Soil and in TNT Finished Compost (21-day exposures. Means and (SD))

Treatment	Survival %[a]	Growth Net Change in Dry Weight, mg
Control soil	100	-19.0 (0.9)
TNT-spiked soil	100	-13.0 (0.8)
Control finished compost	96	77.1 (16.6)
TNT finished compost	35	Not determined

[a] Percent survival to Day 8. Terminated at that time due to high mortality.

The effects of composting could not be determined in the RDX and HMX exposures due to high mortality in the control finished composts. Mortality was 100 percent in both the HMX finished compost and its control finished compost. Mortality was only 5 percent in the RDX finished compost, but 25 percent in its control finished compost. Table 4 shows the effects on growth and reproduction at a concentration of approximately 400 ppm in the RDX- and HMX-spiked soils. Adult worms lost weight in both explosive-spiked soils, while those in the control soil gained weight; but the weight loss was significant for RDX only (Table 4). In all three adult growth measurements (net dry weight change, total biomass dry weight, and total biomass net dry weight change), worms in the RDX-spiked soil were significantly lower than the controls. While these parameters are also negative

Table 4
Effects on Growth and Reproduction of Earthworms, *Eisenia foetida*, of RDX- and HMX-Contaminated Soils (400 ppm) in 21-Day Exposures (Analysis of variance and means comparisons (Tukey's Test))

Test Parameter	Treatment	Mean	N	F	P	Tukey Group[a]
Total biomass dry weight, mg	Control soil	201	60	4.19*[b]	0.0200	A
	HMX-spiked soil	181				AB
	RDX-spiked soil	169				B
Total biomass net dry weight change, mg	Control soil	11.6	60	5.55*[b]	0.0063	A
	HMX-spiked soil	(-) 8.8				AB
	RDX-spiked soil	(-) 18.2				B
Adult net dry weight change, mg	Control soil	6.69	60	4.39*[b]	0.0168	A
	HMX-spiked soil	(-) 10.1				AB
	RDX-spiked soil	(-) 19.1				B
Cocoon dry weight, mg	Control soil	2.68	27	1.02	0.3744	A
	HMX-spiked soil	2.24				A
	RDX-spiked soil	2.74				A
Number of cocoons	Control soil	1.85	60	9.22*[c]	0.0003	A
	HMX-spiked soil	0.5				B
	RDX-spiked soil	0.4				B

Note: *ANOVA significant, $P \leq 0.05$.
[a] Treatment means with the same letter designation are not statistically different, $P \leq 0.05$.
[b] Failed test for normality and/or homogeneity of variances. ANOVA onrankits used.
[c] Failed test for normality and/or homogeneity of variances. Square root transformation used.

for the HMX-spiked soil exposures, they were not statistically different from either the controls or the RDX-spiked exposures.

Both explosives severely impaired reproduction. The mean number of cocoons recovered were fourfold to fivefold fewer in the explosives-exposed than in the control worms (Table 4). In actual numbers, the 20 control soil worm pairs produced 37 cocoons after 21 days, and the same number of RDX- and HMX-spiked soil worms produced only 8 and 10 cocoons, respectively. Dry weights of the cocoons did not differ among the three groups.

Avoidance behavior

Worms avoided the positive control as expected (Table 5). All worms in the TNT-spiked soil exposure were found on the artificial soil side of the dish. The avoidances in both cases were statistically significant. The control soil was preferred to artificial soil 82 percent:18 percent, but the effect was not statistically significant. The worms avoided both TNT and control finished composts in favor of the artificial soil, indicating that the compost itself rather than any TNT metabolite present was disagreeable. Although TNT could not be detected in the TNT finished compost, when given a choice between that and the control finished compost, 68 percent of the worms avoided the TNT finished compost; the effect was statistically significant. The aversion shown to the TNT finished compost suggests that metabolites of TNT also are stress inducing in the worms. In contrast to TNT, the worms showed little aversion when given the choice between RDX- or HMX-spiked soil and artificial soil.

Table 5
Results of 4-Day Earthworm Avoidance Bioassays of TNT-, RDX-, and HMX-Spiked Soils and TNT and Control Finished Composts (Comparison of percentage of worms in artificial soil (negative control) versus percentage in test media (spiked soils and compost))

Treatment	Worms in Artificial Soil, %	Worms in Test Medium, %
TNT-spiked soil	100	0*
Control soil	18	82
KCl- spiked soil (positive control)	94	6*
Artificial soil (negative control)	44	56
TNT finished compost	73	27*
Control finished compost	64	36*
KCl-spiked soil (positive control)	96	4*
Artificial soil (negative control)	48	52
RDX-spiked soil	59	41
KCl-spiked soil (positive control)	77	23*
Artificial soil (negative control)	61	39
HMX-spiked soil	60	40
KCl-spiked soil (positive control)	77	23*
Artificial soil (negative control)	61	39

Note: *Avoidance statistically significant, $P \leq 0.05$ (one-tailed binomial test).

Metabolism and oxidative stress

The primary route by which detoxication of neutral foreign compounds is achieved in eukaryotes is by metabolism and excretion of the conjugated metab olite. In most cases, the xenobiotic is bioactivated by insertion of molecular oxygen catalyzed by mixed-function oxidases of the cytochrome P-450 system (Phase I), enabling it to bind with an endogenous substrate such as glutathione or sulfate (Phase II) (Figure 5, (1)). Some chemical classes such as nitro- or azo-aromatics are reductively bioactivated by the P-450 system, and TNT, RDX, and HMX may be among those. Others, e.g., esters, are hydrolyzed. In all cases, the increased water solubility of the product facilitates excretion. How ever, bioactivation can also result in formation of reactive intermediates capable of covalently binding with macro-molecules such as enzymes or nucleic acids.

Bioactivation may also result in a compound capable of undergoing redox cycling, or in the case of compounds such as quinones, aromatic nitro compounds, or hydroxylamines, the parent compound itself may redox cycle (Figure 5, (2)). Redox cycling is a secondary metabolic pathway in which the parent compound is reduced by flavoproteins transforming it into a radical metabolite (Figure 5, (3)). The radical metabolite is capable of reacting directly with molecular oxygen pro duc-ing the superoxide anion, $O_2^{\cdot-}$, and regenerating the parent compound (Fig ure 5, (4)). The cycle may be repeated with each turn producing another superoxide anion until the metabolite radical is either adducted to an endogenous macro molecule (e.g., DNA or an enzyme) or is conjugated and eliminated (detoxified). Some com pounds can form reactive metabolites by both the cytochrome P-450 and redox cycling routes.

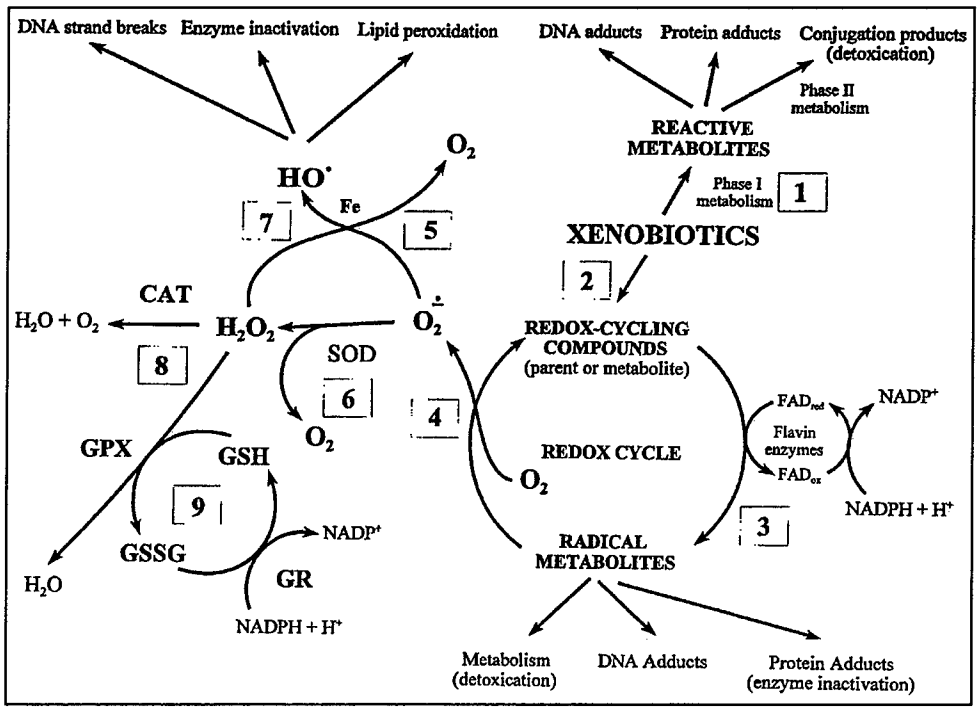

Figure 5. Overview of routes by which xenobiotics may result in toxicity and how they may be detoxified ((1) Phase I metabolism (including oxidation by cytochrome P-450) may form reactive metabolites, which an either react with cellular components or be detoxified by Phase II metabolism (conjugation). (2) Phase I metabolism can also result in the formation of redox cycling compounds. (3) Redox cycling compounds can be reduced by flavoproteins to form radical metabolites. (4) To complete the cycle, the radical metabolite autooxidizes forming the superoxide anion ($O_2^{\cdot-}$) and regenerating the parent compound. (5) In the presence of iron, $O_2^{\cdot-}$ undergoes the Haber-Weiss reaction, forming the highly reactive hydroxyl radical ($HO^{\cdot}$). (6) Superoxide can be degraded by superoxide dismutase (SOD) forming hydrogen perioxide (H_2O_2). (7) In the presence of iron, H_2O_2 can undergo Fenton's reaction forming molecular oxygen and hydroxyl radical. (8) H_2O_2 is degraded by catalase (CAT) or by glutathione peroxidase (GSX), which utilizes reduced glutathione (GSH) as a proton donor. (9) Glutathione reductase (GR) regenerates the protective free thiol (GSH) from glutathione disulfide (GSSG). Details in text) (Adapted from Kappus 1987)

The superoxide radical itself is a poor oxidant and will not result in oxida tive damage to the organism, but in the presence of iron it can undergo the Haber-Weiss reaction, forming molecular oxygen and the extremely reactive hydroxyl radical, $HO^{\cdot}$ (Figure 5, (5)). Superoxide dismutase can reduce the formation of $HO^{\cdot}$ from superoxide radicals by catalyzing the formation of H_2O_2 instead (Figure 5, (6)). Hydrogen peroxide is also capable of forming $HO^{\cdot}$ in the presence of iron through Fenton's reaction (Figure 5, (7)). However, the enzymes CAT and GPX/HP normally function as a defense against this reaction by reducing H_2O_2 to water (Figure 5, (8)). In the case of GPX/HP, GSH acts as a proton donor, resulting in the formation of GSSG (Figure 5, (9)); GR restores GSSG to the protective free thiol.

The importance of redox cycling as a bioactivating process is that each mole of xenobiotic can potentially produce multiple moles of active oxygen species and result in oxidative stress to the organism. Damage begins to accumulate when the formation of active oxygen species exceeds the organism's capacity to neutralize them. This includes mutations that may eventually lead to the forma tion of cancers. Organisms normally cope with increased oxidative stress by increasing the levels of protective enzymes and antioxidants. For this reason, enzymes such as CAT, SOD, GPX, GR, and GST and the substrate GSH may serve as biomarkers.

Effects on oxidative stress enzymes

Selenium-dependent glutathione peroxidase (GPX/HP) activity was significantly lower in worms exposed to the TNT-spiked soil as compared with those in the control soil, indicating a reduced capacity to metabolize H_2O_2 (Table 6). No other enzymes in worms exposed to TNT-spiked soil were significantly different from those of their respective controls, although differences were observed between

Table 6
Effects on Oxidative Stress Enzymes in Earthworms, *Eisenia foetida*, of TNT-Spiked Soil and Control and TNT Finished Composts in 21-Day Exposures (Analysis of variance and means comparisons (Tukey's Test))

Test Parameter[a]	Treatment	Mean	N	F	P	Tukey Group[b]
Superoxide dismutase (SOD)	Control soil	0.109	35	0.31[c]	0.8203	A
	TNT finished compost	0.108				A
	Control finished compost	0.102				A
	TNT-spiked soil	0.097				A
Catalase (CAT)	TNT-spiked soil	4.789	30	4.03*	0.0156	A
	Control soil	4.086				AB
	TNT finished compost	3.962				AB
	Control finished compost	2.856				B
Se-dependent glutathione peroxidase (GPX/HP)	TNT finished compost	6.124	30	4.71*	0.0094	A
	Control soil	5.495				A
	Control finished compost	5.520				AB
	TNT-spiked soil	4.458				B
Se-independent glutathione peroxidase (GPX/CP)	TNT-spiked soil	2.987	30	0.28	0.8419	A
	TNT finished compost	2.866				A
	Control soil	2.787				A
	Control finished compost	2.682				A
Glutathione-S-transferase (GST)	Control soil	386	30	2.71[c]	0.0653	A
	TNT-spiked soil	352				A
	TNT finished compost	257				A
	Control finished compost	201				A
Glutathione reductase (GR)	Control finished compost	3.859	35	4.06*	0.0152	A
	TNT finished compost	3.636				AB
	TNT-spiked soil	2.619				AB
	Control soil	2.303				B

Note: *ANOVA significant, $P \leq 0.05$.
[a] Enzyme units are: SOD: mg postmitochondrial protein for 50-percent inhibition of cytochrome c oxidation in the presence of superoxide produced by the xanthine:xanthine-oxidase system. CAT: (ln(A1/A2))/((T2-T1)*mg cytosolic protein). GPX/HP and GPX/CP: µmol NADPH consumed/min*mg postmitochondrial protein. GST: µmol CDNB conjugated/ min*mg cytosolic protein. GR: µmol NADPH consumed/min*mg cytosolic protein.
[b] Treatment means with the same letter designation are not statistically different, $P \leq 0.05$.
[c] Failed test for normality and/or homogeneity of variances. ANOVA onrankits used.

Table 7
Effects on Oxidative Stress Enzymes in Earthworms, *Eisenia foetida*, of RDX- and HMX-Spiked Soils in 21-Day Exposures (Analysis of variance and means comparisons (Tukey's Test))

Test Parameter[a]	Treatment	Mean	N	F	P	Tukey Group[b]
Superoxide dismutase (SOD)	Control soil RDX-spiked soil HMX-spiked soil	0.097 0.089 0.119	30	4.65*	0.0183	AB B A
Catalase (CAT)	Control soil RDX-spiked soil HMX-spiked soil	4.69 3.85 3.92	30	1.02	0.3749	A A A
Se-dependent glutathione peroxidase (GPX/HP)	Control soil RDX-spiked soil HMX-spiked soil	4.7 4.4 4.4	30	0.12	0.8849	A A A
Se-independent glutathione peroxidase (GPX/CP)	Control soil HMX-spiked soil RDX-spiked soil	1.7 1.6 1.3	30	0.86	0.4349	A A A
Glutathione-S-transferase (GST)	Control soil HMX-spiked soil RDX-spiked soil	317 277 247	30	0.33	0.7241	A A A
Glutathione reductase (GR)	Control soil HMX-spiked soil RDX-spiked soil	3.74 2.90 2.80	30	4.53*	0.0201	A B B

Note: *ANOVA significant, $P \leq 0.05$
[a] Enzyme units are: SOD: mg postmitochondrial protein for 50-percent inhibition of cytochrome c oxidation in the presence of superoxide produced by the xanthine:xanthine-oxidase system. CAT: (ln(A1/A2))/((T2-T1)*mg cytosolic protein). GPX/HP and GPX/CP: µmol NADPH consumed/min*mg postmitochondrial protein. GST: µmol CDNB conjugated/min*mg cytosolic protein. GR: µmol NADPH consumed/min*mg cytosolic protein.
[b] Treatment means with the same letter designation are not statistically different, $P \leq 0.05$.

control soil and control finished compost exposures. This lack of induction of protective enzymes combined with a decrease in GPX/HP activity may reduce the ability of the earthworms to protect themselves from oxidative stress.

The only significant difference observed in enzyme responses between HMX- or RDX-spiked soil and control soil exposures was a decrease in glutathione reductase activity, which may be indicative of a toxic response (Table 7).

Protein

Little or no effect on either postmitochondrial or cytosolic protein was observed in TNT-exposed animals (Table 8). The difference in means in the Tukey test between the TNT-spiked soil worms and the TNT finished compost should be regarded cautiously, as the variances remained unequal even after transformation by rankits. No significant effects on protein content were observed upon exposure to RDX. However, exposure to HMX resulted in significantly reduced postmitochondrial protein relative to control. As both SOD and GPX activity are normalized to the postmitochondrial protein, the overall ability of the worms to protect against

oxidative stress via these enzymes (activity per gram of worm) is further decreased. Although SOD activity was not significantly different in control and HMX-exposed animals, the slightly higher value for HMX-exposed worms indicates that the activity of the enzyme was slightly lower (it takes more protein to inhibit the superoxide-driven reaction). Combined with a decrease in overall protein content in the animals, the ability of SOD to protect the worms from superoxide radicals is most likely lower in animals from the HMX treatment as compared with those of the control.

Glutathione

In the TNT-spiked soil exposures, the total glutathione (GSH + GSSG) and the percent GSSG were not significantly different from that of the control (Table 9). However, the amount of GSSG present was markedly greater in the TNT-exposed than in control worms ($P = 0.007$), indicating that the organisms were under increased oxidative stress. That the HMX-exposed organisms were also under increased oxidative stress is indicated by their significantly higher total glutathione content relative to that of control worms; i.e., the HMX-exposed worms were induced to synthesize additional glutathione in order to cope with the toxicant (Table 10). The interpretation that the TNT- and HMX-exposed worms were under increased oxidative stress is also supported by the fact that compared with their respective control treatments, GPX/HP activity in TNT-exposed worms decreased (Table 6). Furthermore, the overall ability to detoxify oxidants in HMX-exposed worms was reduced due to the decreased postmitochondrial protein content (Table 8) and the reduced GR activity (Table 7). Exposure to RDX did not significantly affect total glutathione content or postmitochondrial protein content, and of the antioxidant enzymes only GR was significantly lower than control (Table 7). Although GR was significantly reduced relative to control in the RDX-spiked soil exposure, neither total glutathione nor the amount of GSSG present differed from control (Table 10). These results suggest that oxidative stress does not play a role in RDX toxicity, or at most only a minor one. The GR reduction may have been a nonspecific indicator of toxicity that resulted from effects on other systems.

Immunotoxicity

In the TNT-spiked soil exposures, the number of coelomocytes extruded, and the percentage of the cells that were dead were not significantly different from the controls (Table 11). However, a significant decrease in the ability of the worms to reduce nitroblue tetrazolium (NBT) in the TNT-spiked soil indicated that the worms' immune response had been compromised. Too few worms survived in the TNT finished compost for comparisons to be made. The impact on immunocompetence of the worms was even greater in the RDX-spiked soil with significantly fewer cells being extruded than in the controls, a greater although nonsignificant percentage of extruded cells that were dead, and a severe reduction in the worms' ability to reduce NBT ($P = 0.0001$). HMX also exhibited immunotoxic potential, as the overall amount of cells extruded was significantly lower in HMX-exposed worms as compared with that of the control.

Table 8
Effects on Protein in Earthworms, *Eisenia foetida*, of TNT-, HMX-, and RDX-Spiked Soils and TNT and Control Finished Composts in 21-Day Exposures (Analysis of variance and means comparisons (Tukey's Test))

Test Parameter	Treatments	Mean	N	F	P	Tukey Group[a]
Cytosolic protein, mg g^{-1} earthworm, wet wgt	TNT finished compost	3.11	35	2.86[bc]	0.0526	A
	Control finished compost	2.62				AB
	Control soil	2.45				AB
	TNT-spiked soil	2.27				B
	HMX-spiked soil	2.27	30	0.21	0.818	A
	Control soil	2.21				A
	RDX-spiked soil	2.12				A
Postmitochondrial protein, mg g^{-1} earthworm, wet wgt	TNT finished compost	3.56	30	2.40	0.091	A
	Control finished compost	3.19				A
	Control soil	3.09				A
	TNT-spiked soil	2.88				A
	Control soil	3.51	30	4.07*	0.0284	A
	RDX-spiked soil	3.25				AB
	HMX-spiked soil	2.96				B

Note: *ANOVA significant, $P \leq 0.05$.
[a] Treatment means with the same letter designation are not statistically different, $P \leq 0.05$.
[b] Failed test for normality and/or homogeneity of variances. ANOVA onrankits used.
[c] Variances unequal even after transformation.

Table 9
Effect on Glutathione in Earthworms, *Eisenia foetida*, of TNT-Spiked Soil After 5 Days of Exposure (T-test on means)

Test Parameter	Treatment	Mean	Degrees of Freedom	T	P
Total glutathione (GSH + GSSG), µmol g^{-1} tissue wet wgt	Control soil	0.863	8	0.609	0.559
	TNT-spiked soil	0.754			
Oxidized glutathione (GSSG), µmol g^{-1} tissue wet wgt	TNT-spiked soil	0.640	4	5.175*	0.007
	Control soil	0.390			
Percent GSSG	TNT-spiked soil	6.500	4	0.933	0.277
	Control soil	5.567			

Note: *Significant, $P \leq 0.05$.

Genotoxicity

The TNT-spiked soil was not genotoxic to the worms, but DNA breaks were significantly greater (lower mean F-value) in the TNT finished compost than in soil, indicating that composting TNT-spiked soil caused it to become genotoxic (Table 12). Neither RDX nor HMX were genotoxic, with F-values virtually identical to the control worms. Chemical analysis of the TNT finished compost indicated the presence of 4.55 mg 4ADNT kg^{-1} (Table 1). These results are consistent with

Table 10
Effects on Glutathione in Earthworms, *Eisenia foetida*, of HMX- and RDX-Spiked Soils After 21-Day Exposures (Analysis of variance and means comparisons (Tukey's Test))

Test Parameter	Treatment	Mean	N	F	P	Tukey Group[a]
Total glutathione (GSH + GSSG), µmol g^{-1} tissue wet wgt	HMX-spiked soil RDX-spiked soil Control soil	0.701 0.557 0.553	30	4.40*	0.0222	A B B
Oxidized glutathione (GSSG), µmol g^{-1} tissue wet wgt	HMX-spiked soil RDX-spiked soil Control soil	0.046 0.038 0.038	30	0.93	0.4087	A A A
Percent GSSG	HMX-spiked soil RDX-spiked soil Control soil	7.84 6.85 6.33	30	2.79	0.0791	A A A

Note: *ANOVA significant, $P \leq 0.05$.
[a] Treatment means with the same letter designation are not statistically different, $P \leq 0.05$.

Table 11
Immunotoxicity to Earthworms, *Eisenia foetida*, of TNT-, RDX-, and HMX-Spiked Soils in 21-Day Exposures (Analysis of variance and means comparisons (Tukey's Test))

Test Parameter	Treatment	Mean	N	F	P	Tukey Group[a]
Number of extruded cells, ml^{-1}	TNT-spiked soil Control soil	14.88×10^6 10.68×10^6	10	2.95	0.1243	A A
	Control soil RDX-spiked soil	7.076×10^6 4.662×10^6	20	13.23*	0.0019	A B
	Control soil HMX-spiked soil	5.510×10^6 4.315×10^6	20	12.62*[b]	0.0023	A B
Dead cells, percent	Control soil TNT-spiked soil	19.32 16.22	10	0.89	0.3723	A A
	RDX-spiked soil Control soil	25.9 23.8	20	0.40	0.5363	A A
	HMX-spiked soil Control soil	26.2 22.6	20	2.78[b]	0.1129	A A
NBT reduction assay (absorbance, 515 nm)	TNT-spiked soil Control soil	0.0690 0.0566	10	7.16*	0.0281	A B
	Control soil RDX-spiked soil	0.0815 0.0740	20	37.23*[b]	0.0001	A B
	Control soil HMX-spiked soil	0.0685 0.0660	20	0.26[b]	0.6187	A A

Note: *Statistically significant, $P \leq 0.05$.
[a] Treatments with the same letter designation are not statistically different, $P \leq 0.05$.
[b] Failed test for normality and/or homogeneity of variances. ANOVA onrankits used.

Table 12
Effects on DNA Single-Strand Breaks in Earthworms, *Eisenia foetida*, of TNT-, RDX-, and HMX-Spiked Soils and TNT and Control Finished Composts in 21-Day Exposures (Analysis of variance and means comparisons (Fisher's Least Significant Difference Test))

Test Parameter	Treatment	Mean	N	F	P	LSD Group[a]
SsDNA, F-value	Control soil	0.661	30	3.78*[b]	0.0224	A
	TNT-spiked soil	0.637				A
	Control finished compost	0.632				A
	TNT finished compost	0.425				B
	Control soil	0.676	30	0.15	0.8578	A
	HMX-spiked soil	0.649				A
	RDX-spiked soil	0.646				A

Note: *ANOVA significant, $P \leq 0.05$.
[a] Treatment means with the same letter designation are not statistically different, $P \leq 0.05$.
[b] Failed test for normality and/or homogeneity of variances. ANOVA onrankits used.

previous research in which 4ADNT and other breakdown prod ucts of TNT were found to be mutagenic in bacterial assays (Honeycutt, Jarvis, and McFarland 1996).

Conclusions

TNT-spiked soil (approximately 200 mg kg^{-1}) and both the finished TNT compost and control finished compost invoked avoidance responses in the earthworms. When forced to choose between the control and TNT finished composts, the earthworms chose the former. Because TNT in the TNT finished compost was below analytical detection limits, and because the TNT metabolite 4ADNT was found, the most probable cause of avoidance was the metabolite. Neither RDX- nor HMX-spiked soil (approximately 400 mg kg^{-1} each) was avoided by the worms. TNT-spiked soil and HMX-spiked soil both provided evidence of oxidative stress effects on glutathione, antioxidant enzymes, and postmitochondrial protein. RDX-spiked soil did not do so. The finished TNT compost had no evident effect on the antioxidant enzymes. Insufficient tissue was available due to high mortality in the finished TNT compost to allow assays for glutathione and immunocompetence. TNT-spiked soil and RDX-spiked soil reduced the immunocompetence of the worms. HMX also appeared to reduce immunocompetence, but had a lesser effect. The TNT-, HMX-, and RDX-spiked soils were nongenotoxic as measured by DNA single-strand breakage; but the finished TNT compost was strongly genotoxic, and this result was consistent with the presence of 4ADNT. Although RDX and HMX both appear to cause several sublethal toxic responses, neither compound caused mortality or genotoxicity, and neither were avoided by the earthworms. How ever, despite the lack of acute toxicity, both explosives reduced growth of the adults and severely impacted reproduction. These chronic toxicities have the potential to adversely affect ecosystems at the population level and have not previously been identified as consequences of the exposure of organisms to RDX or HMX.

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6 Conclusions

Character of Immobilized Products

An understanding of the chemical interactions between TNT transformation products and functional groups on humic substances was achieved using liquid- and solid-state ^{15}N NMR analysis. Results indicated that the mono and diamino transformation products of TNT covalently bind to carbonyl compounds, humic substances, and whole peat in compost and soil. The number of potential prod ucts forming from the interaction of the diamino transformation products of TNT with humic functional groups is greater than the number from the mono amino transformation products. This is attributed to the higher pKa and greater nucleophilicity of the diamino products. Furthermore, the 4ADNT produces more products than the 2ADNT. This is due to steric hindrance effects in the 2ADNT.

The enyme peroxidase failed to catalyze auto condensation of the mono amines. However, the enzyme catalyzed the formation of several products from 2,4DANT, some identified (e.g., azoxybenzenes, imines, hydrazines) and some uncertain. Fewer products were observed from 2,6DNT. Polymerization of conniferyl alcohol by peroxidase is a potential biosynthetic pathway for the forma tion of lignin in woody plants. Peroxidase catalyzed reactions of the mono and diamines with conniferyl alcohol resulting in complex products. These results demonstrate the possibility of such reactions in plants.

Reactions between the diamines and humic acid in the presence of the enzyme resulted in greater imine formation than heterocyclic nitrogen formation. Deamination with release of ammonia was also observed. In the absence of the enzyme, the relative proportion of imines and ammonia were less. Reactions between the monoamines and humic acid results in more heterocyclic products than imines. The enzyme also enhanced these reactions.

Spectral analyses before and after hydrolysis of TNT that had been reacted with peat indicated that some bonds were not hydrolyzable. Aminoquinone, amide, and imine linkages were hydrolyzable, while aminohydroquinone bonds persisted. Heterocyclic products were resistant to hydrolysis.

The products of the interactions between TNT transformation products and humic substances are complex. The progression of covalent bonding reactions

results in products exhibiting various potentials for subsequent release. How ever, a significant proportion of these reactions are extremely stable. Therefore, long-term stability of the products of these reactions, and consequently of the effectiveness of the remediation processes in which they occur, is a function of the local environment, the transformation products available for reaction, and the progression of the reactions.

Chemical Stability and Analytical Methods

The transformation of TNT to mono and diamino transformation products and the subsequent reactions of these products with organic material in compost and digester sludge occurs in two stages. After the first few days of compost or digester sludge treatment, about 20 percent of the transformed TNT was released by hydrolysis as identifiable transformation products. After about 40 days, very little transformed TNT was released from either system. Current analytical methods of solvent extraction are effective in removing unconjugated TNT and TNT transformation products from these treatment matrices. The hydrolysis regime developed in this study is effective in removing the weakly conjugated amines that are subject to further reaction. This pool constitutes the potentially available products resulting from treatment at the stage where the analysis is conducted. When treatment takes the reaction sequence to completion, no hydrolyzable products are released. The hydrolysis method can, therefore, serve as an indicator of treatment progression and/or effectiveness. The method is not sufficiently sensitive for application to immobilization processes in soils due to the relatively small amount of organic matter in soils.

Microbial Degradation

Composted TNT-contaminated soils were not degrad able by microorganisms. Very few microorganisms were able to grow on the compost because it failed to provide sufficient carbon and energy. When the most degradable organic fraction, cellulose, was subjected to degradation studies independently of the rest of the compost matrix, microorganisms were able to mineralize significant quantities of added radiolabeled TNT (up to 23 percent). Limited mineralization activity was also observed in the fulvic acid fraction. These results demonstrate the limits of degradation potential in finished com post. Since these fractions are dispersed in the compost matrix, their degradation potential in whole compost is extremely small. Therefore, release of environmentally hazardous products from finished compost by microbial action is unlikely.

Toxicity

Results of Mutatox and Ames assays of TNT and the amino transformation products indicated no to moderate toxicity. In general, the monoamino transformation products of TNT were equally or only slightly less toxic than the parent

compound. However, toxicity of the compounds are best assessed in tests that take bioavailability into account. Therefore, a battery of earthworm bioassays on TNT-contaminated compost and soils were conducted. The battery included acute toxicity, chronic toxicity, avoidance behavior, metab olism and oxidative stress, immunotoxicity, and genotoxicity. The 14-day LC_{50} in soil was 353 mg kg^{-1}. Attempts to define similar toxicity for RDX and HMX were abandoned at soil concentrations of 4,000 mg kg^{-1} when no earthworms died. Earthworms exhibited high chronic toxicity to finished TNT compost, 35 -percent survival compared with 96-percent for controls. This toxicity was attributed to the 4ADNT rather than to TNT because no TNT was extractable from the matrix while 4ADNT was found at 4.55 mg kg^{-1}. Earthworms avoided the finished compost, suggesting that colonization of finished compost by earthworms is not likely. The residual TNT transformation products may induce stress in the worms causing avoidance. Oxidative stress effects on several enzymes and protein were observed in worms exposed to TNT-contaminated soils and compost. TNT-contaminated soils reduced immunocompetence in the worms, but was not genotoxic as measured by DNA single-strand breakage. However, finished compost was highly genotoxic. This result was attributed to the presence of extractable transfor mation products of TNT.

These results suggest that the relative completion of the immobilization process when composting is terminated will dictate the availability of TNT residuals for toxic effects on earthworms. If amino transformation products are extractable, the likelihood of residual toxicity in the compost is greater than if these products are absent.

Summary

An understanding of the processes by which TNT becomes immobilized in remediation systems such as compost has been achieved. The chemical nature of the immobilization products and some of the factors controlling the character of these products have been defined. The long-term stability of the products of immobilization have been explored in terms of their susceptibility to chemical hydrolysis, microbial degradation potential, and toxicity. An analytical method for taking a snapshot of the evolving products of the immobilization process has been developed. Results of this research will contribute to the optimization and regulatory acceptance of composting. Results also have important implications for other biotreatment processes including natural attenuation.

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Immobilization of 2,4,6-trinitrotoluene (TNT) in soils and in compost had been widely documented. Dramatic reductions in solvent extractable TNT have been observed, while complete recoveries of radioactivity from carbon-14 labeled TNT indicated that all of the products of TNT remained in the matrix in some form. The objectives of these studies were to determine the nature and mechanism of immobilization and the long-term stability, biodegradability, and toxicity of the final products. The approach included ^{15}N-nuclear magnetic resonance analysis of binding to humic materials, acid/base hydrolysis with subsequent identification of products, microbial degradation, and toxicity studies. Results confirm covalent bonding of the amino transformation products of TNT that is only slightly reversible, depending upon the degree of comple tion to which the reactions have occurred. Products were resistant to microbial degradation and relatively stable against hydrolysis. Toxicity was related to bioavailability, which depended upon the degree of completion of the immobilization reactions. These results have important implication for fate of TNT in remediation systems and in the environment.

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